Universidad Carlos III de Madrid

TRABAJO FIN DE GRADO

The effect of the UVB light in epidermal cells of Xeroderma Pigmentosum-C

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ACKNOWLEDGMENTS

Gracias a mis padres por estar siempre ahí, para que no me desanimase. Que con paciencia y esfuerzo todo se consigue.

Gracias Marta por hacer que esto fuera posible.

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1.ABSTRACT

The experimental work of the bachelor thesis was focused on the application of the knowledge acquired in Tissue Engineering, Regenerative Medicine and Biomaterials; which are branches within Biomedical Engineering. Particularly, it is concentrated on the study of rare skin diseases.

The project is divided in two experimental studies. The first is centered in the study of cytotoxic effects of UV radiation, specifically UV type B (UVB), over a photosensitive skin disorder named Xeroderma Pigmentosum.

The second experimental study goes a step further, in order to achieve an adhesion assay over different biomaterials to evaluate cell growth and cell attachment to these different matrices.

2.MOTIVATION AND OBJECTIVES

As the skin is the outermost tissue of the human body, it represents the shield against environmental stress. There are different sources which damage the skin; however Ultraviolet (UV) radiation is one of the most harmful to the skin. In this way there are skin disorders affecting people throughout the world. These diseases range from some very common such as dermatitis or acne to rare and lethal ones such as Xeroderma Pigmentosum type C (XPC).

Individuals with Xeroderma Pigmentosum have extreme photosensitivity to UV light. This photosensitivity is intended to be studied by inducing an acute damage to XPC cells using a controlled source of UVB radiation.

In addition, an adhesion study over different matrices of natural origin is performed trying to find which one is the best to serve as a scaffold to a possible treatment.

3.PLANNING

- **PHASE 1 (OCTOBER 2013). SELECTING A RESEARCH TOPIC AND LITERATURE SEARCH.** At the very beginning a deep information research was performed, based on the knowledge acquired along the university years.

- **PHASE 2 (2nd WEEK NOVEMBER 2013). MENTOR MEETING.** In the first reunion, the aim was to determine the topic to be investigated and the existing experimental limitations; then an organization of the development of the experiment was also established.

- **PHASE 3 (3rd WEEK NOVEMBER 2013 UNTIL FIRST WEEK DECEMBER 2013). MATERIAL PREPARTION AND EXPERIMENTAL DEVELOPMENT.** All the materials needed to develop the experiments, including the used cells and cell culture media were prepared to allow the development of the assays. At this time, the first irradiation study was performed.

- **PHASE 4 (2nd WEEK DECEMBER 2013). RESULTS ANALYSIS AND DISCUSSION WITH THE TUTOR.** With the obtained results, an analysis was meticulously performed and they were discussed with the project´s mentor. A possible continuation of the experiment was proposed oriented to the adhesion study.

- **PHASE 5 (3rd WEEK DECEMBER 2013, JANUARY 2014 AND FIRST WEEKS FEBRUARY 2014). LITERATURE RESEARCH ABOUT XERODERMA PIGMETOSUM GROUP C.** Due to little available information about Xeroderma Pigmentosum corresponding to the adhesion study; this part lasted two months.

- **PHASE 6 (4th WEEK FEBREARY 2014). MATERIAL PREPARATION FOR XERODERMA PIGMETOSUM GROUP C AND EXPERIMENTAL DEVELOPMENT.** As in the phase 3 all the materials needed to develop the experiments; including the used cells, cell culture media and the matrices were prepared to allow the development of the assays. At this time, a controlled UVB radiation and the adhesion assay was made.

- **PHASE 7. ANALYSIS AND DISCUSSION OF THE OBTAINED RESULTS WITH THE TUTOR.** The obtained results were analyzed and

unexpected results were found due to possible fails along the development of the experiments.

- **PHASE 8 (1st WEEK SEPTEMBER 2014). TUTOR MEETING.** The purpose of the meeting was to determine the steps to follow in the repetition of the adhesion study.

- **PHASE 9 (2nd WEEK SEPTEMBER 2014 UNTIL 2nd WEEK DECEMBER 2014). REPETITION OF THE PREVIOUS EXPERIMENTS.** The previous experiments had not produced the expected results, so they were repeated and new material preparation was required. This stage had a long duration due to problems found in the growth the cells.

- **PHASE 10 (3rd WEEK DECEMBER 2014 UNTIL 1st WEEK OF FEBRUARY 2015). ELABORATION OF THE FINAL MEMORY.** Time inverted in the elaboration of the written document individually.

- **PHASE 11 (2nd AND 3rd WEEKS OF FEBRUARY 2015). FINAL MEETING WITH TUTOR.** This time was employed in making suggestions about possible changes and improvements.

- **PHASE 12 (3rd WEEK OF FEBRUARY 2015) END OF THE MEMORY.** In this last stage final modifications were made and incorporated.

4.INTRODUCTION

4.1.SKIN

The skin is the largest organ of the human body covering the whole external surface with an area of $2m^2$ and accounting for the 16% of total body weight [1]. The most representative function of the skin is to form a physical barrier enabling the entrance and exit of several substances such as water or electrolytes and protecting the body from external pathogens such as bacteria and virus, UV light, toxic sources and mechanical stresses.

The skin is divided into two main layers: the superficial epidermis and the deeper dermis [2] and beneath these two is the subcutaneous layer or hypodermis; as it is shown in figure 1. Fibers of elastin and collagen, originating from the dermis, are anchored to this layer [3]. The hypodermis is attached to the underlying fascia, which is the connective tissue that surrounds bones and muscles. In this layer there are lobules of adipocytes, which are the cells in which the fat is contained and stored, within the connective tissue. In this region, there are present nerve terminations sensitive to pressure (mechanoreceptors), known as pacinian or lamellated corpuscles [4]. In addition, larger blood vessels that supply the skin are also localized in the hypodermis [5].

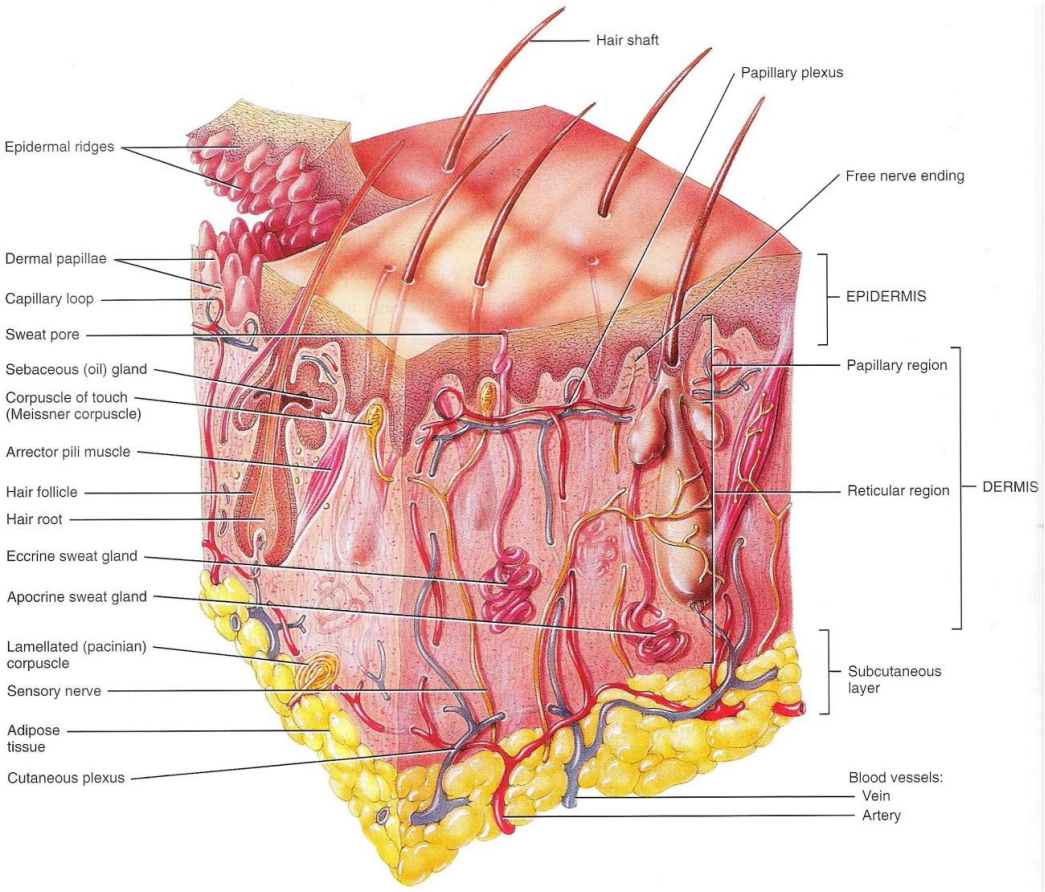

Figure 1: sectional view of skin and subcutaneous tissue

Epidermis

Epidermis is squamous and stratified (multilayered) epithelium and it is the outermost layer separating the interior of the body from the external environment [6]. The epidermis has different thickness based on the part of the body; being 5μ on the eyelids and 1.5 mm in palms and soles [5].The principal epidermal cellular components are keratinocytes. Other types of cells present in the epidermis are: Merkel cells, Langerhans cells and melanocytes; which are briefly described below.

Types of epidermal cells

Merkel cells, are the fewest numerous cells in the epidermis; they are associated to nerve endings of sensory neurons in a characteristic structure called Merkel disc, i.e. they form "synapse-like" contacts [7]. Their function is related to the detection of touch sensations; i.e. they are identified as mechanoreceptors [8, 9].

Langerhans cells are originated in the red bone marrow and then migrate to the epidermis [6]. These cells participate in the immune responses by helping other cells of the immune system in the recognition and elimination of the pathogens that try to penetrate the skin.

Melanocytes play an important role in the production of melanin, which is stored in granules called melanosomes. These cells have thin dendritic projections that are scattered into the keratinocytes to deliver the melanin. Melanin is yellow-red or brown-black pigment which brings about the skin color. It is also responsible of absorbing the Ultraviolet Radiation (UV). The melanin granules are situated inside the keratinocytes, there they are clustered around the nucleus to form like a "veil". In this way, it is believed that DNA is shielded from damage induced by UV light [10].

The most abundant cells in the epidermis are the keratinocytes which are organized in several strata producing different kind of the proteins called keratins. The presence of these proteins confer toughness to the skin which contributes to the protection of the underneath layers [11]. Those strata represent the different stages of differentiation of keratinocytes which constitute the epidermis. The strata are named from the top to the bottom as: stratum corneum, stratum lucidum, stratum granulosum, stratum spinosum and stratum basale. Four of these five layers are present throughout the body; the exception is stratum lucidum which is only localized in those regions which are exposed to high friction.

Stratum basal

Stratum basal is the deepest layer of the epidermis, consisting in a single line of columnar keratinocytes, alternated with the presence of melanocytes, Merkel cells and their corresponding disks. Among the keratinocytes, there are stem cells in continuous cell division to renew the keratinocytes of the above layer. Keratinocytes of the stratum basal synthesize keratin intermediate filament proteins in their cytoskeleton. These filaments allow the cells to be connected to the cells of the stratum spinosum and to the cells of the same row through desmosomes. These are complex molecular structures, being the site in which intermediate filaments are anchored; i.e. they are specialized in cell-to-cell adhesion [6, 12]. In addition, keratinocytes are also attached to the basement membrane through hemidesmosomes, which represent the linkage between intermediate filaments and proteins of the integrin superfamily [6, 13].

Stratum spinosum

Once the basal cells are sufficiently matured, they migrate to the outer layer of the skin and initially form the stratum spinosum. The stratum spinosum is formed by several layers of keratinocytes, of polygonal shape in bottom layers and become flat in the top ones. The cells of this layer produce rougher bundles of keratin filaments that are inserted into desmosomes maintaining cells attached and endowing mechanical strength to the skin [14]. Langerhans cells and projections of melanocytes are also found scattered in this layer, as it can be seen in figure 2.

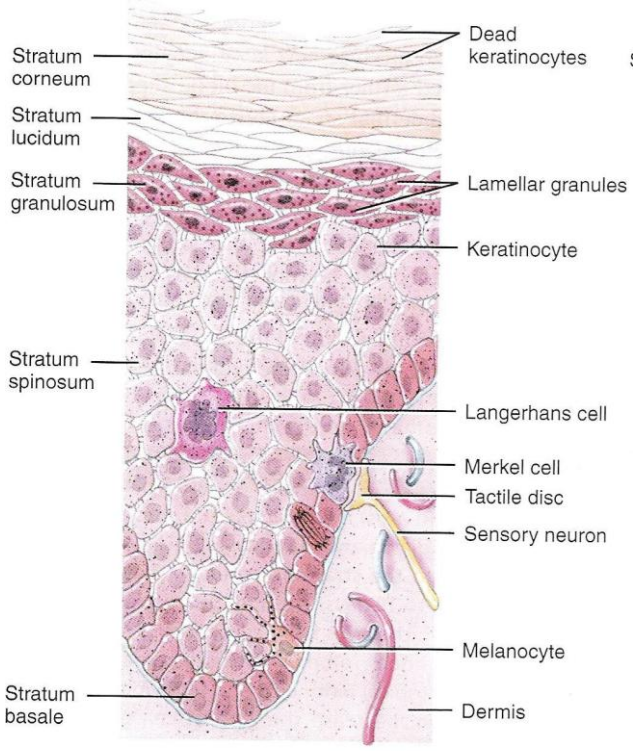

Figure 2: Five principal layers in the epidermis

Stratum granulosum

Above to the suprabasal cell layer lies the stratum granulosum which is an intermediate state where a few layers of keratinocytes continue differentiating. At this level the cells undergo apoptosis (i.e. programmed cell death); their nuclei and their cytoplasmic organelles are degraded by specialized enzymes [15]. As a consequence, their shape is more flattened. This layer is also known as granular cell layer due to the presence of dark granules of keratohyalin which consists of two proteins, profilaggrin and involucrin/filagrin. The first one is the precursor of filagrin, responsible of assembling the keratin filaments into macrofibrils which allow the cross-linking of the intermediate filaments to form a highly insoluble keratin matrix whose function is to attach proteins and lipids in the stratum corneum [16]. The second is a precursor of the cross-linked envelope of cells present in the stratum corneum [17]. In addition there are present other organelles secreted by keratinocytes called lamellar granules, which are responsible of the secretion of a lipid-rich substance that is accumulated in the space among the cells of the stratum granulosum, stratum corneum or stratum lucidum [18].

Stratum lucidum

The stratum lucidum is on top of the granular cell layer in those skin regions which are needed of more toughness as the soles, palms and fingertips. This layer contains three to five rows of dead keratinocytes with high contents of keratin and thickened membranes [19].

Stratum corneum

The final result of the keratinocyte maturation is found at the stratum corneum, which is composed of several sub-layers of flat-like shape corneocytes. These cells are derived from terminally differentiated keratinocytes of the granular layer. Corneocytes are flat, with a plate-like shape and their cytoplasm has enclosed packages of keratin [19]. These cells are protected by a cornified envelope made up of proteins (involucrin/ filaggrin) [20]; a lamellar lipid phase, extruded by lamellar granules of granular cell layer [18], surrounds the space among corneocytes [21]. In addition, they are maintained connected through corneodesmosomes [20]. The final structure represents the natural physical and semi-permeable barrier of the skin [18].

Basement membrane or dermal-epidermal junction

The interface between the stratum basal and the upper part of the dermis (papillary region) is called the basement membrane (BM). It is a composite of several extracellular macromolecules specifically ordered into a matrix called extracellular matrix. It can be clearly distinguished two different layers: the upper is named lamina lucida and the lower is called lamina densa. The lamina lucida is in direct contact to the lipid membranes of keratinocytes from the stratum basal. Beneath the lamina lucida is the lamina densa which is associated to the upper part of the dermis called papillary region [22]. Lamina densa is composed by collagen type IV and other kind of proteins including laminin family. There exists a meshwork formed by attachment structures including hemidesmosomes, anchoring fibrils and anchoring filaments [15]. This network is extended from the intracellular space of basal keratinocytes through their plasma membranes, crossing the dermal-epidermal junction and extending to the papillary region [23]. The main functions of the basement membrane are the maintaining of tissue integrity, serving as a scaffold for cell-to-matrix adhesion and regulation of cell proliferation and differentiation [24].

Hemidesmosomes are complex attachment structures in which many proteins are participants. Their structure is extended from the intracellular space of basal keratinocytes to the lamina lucida, as it can be observed in the figure 3. The major components of this complex are integrins which are transmembrane proteins consisting into a dimer of α and ß subunits [25]. These proteins are located in the plasma membrane of basal keratinocytes, where they mediate the binding of these cells to laminin proteins of the basement membrane. In this way, integrins stabilize the attachment of basal keratinocytes to the basement membrane [19].

Laminins are a family of proteins present in the basement membrane; consisting of three α, ß and γ chains which form the twisted characteristic shape, as can be seen in figure. The principal laminin components present in skin are: laminin 5, also called laminin 332; laminin 6 or 311; laminin 10 or 511; and laminin 1 or laminin 111 [19].

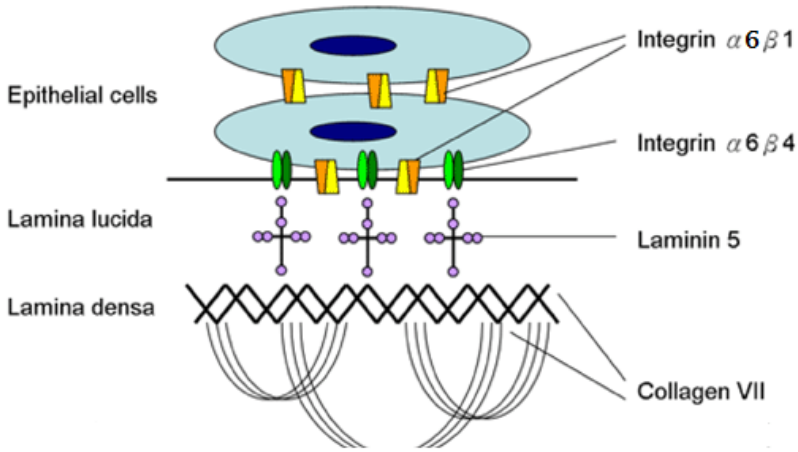

Figure 3: Distribution of the different molecules in BM

Dermis

The deeper and thicker part of the skin beneath the basement membrane is the dermis, which is composed of dense irregular connective tissue forming a complex network [11]. The structures embedded in the network are: collagen and elastic fibers conferring to the skin its characteristic strength, elasticity and resilience; ground substance which is an amorphous gel whose components are primarily glycosaminoglycans providing hydration to the skin [15, 19]. In the dermal structure two sub-layers can be distinguished: the upper part called papillary region and the lower part called reticular region.

The papillary region is constituted by thin collagen and elastic fibers which are not precisely arranged. There are small structures which are projected in the beneath surface of the epidermis with "nipple-like" shape known as dermal papillae. These structures may contain blood vessels; Meissner corpuscles which are receptors of tactile sensations; and free nerve endings responsible of sensations such as coolness, tickling or pain [26].

The reticular region which is connected to the hypodermis is composed of dense packages of thick collagen fibers precisely organized forming a network [15]. There are also present fibroblasts, scattered macrophages and elastic fibers. Since the subcutaneous layer is below, some adipocytes may be found in the deepest part. In this part, blood vessels, nerves, sweat and oil glands are embedded within the meshwork [27].

Human skin is almost constantly subjected to environmental stress and aggression. Sunlight, particularly in the form of UV radiation, is known to be one of the most important examples of agents affecting the skin. Solar radiation causes damage to the DNA.

4.2.SOLAR RADIATION, UV LIGHT TYPES AND EFFECTS

Solar light is electromagnetic radiation of continuous spectrum that can be divided into main three components: ultraviolet (5.4 percent), visible (67.2 percent) and infrared (31.9 percent) reaching the earth from the sun [28]. The ultraviolet (UV) light portion is distributed between 100-400 nm. Particularly UV radiation is composed of three bands, that can be categorized according to the different wavelengths: UVC (100-280 nm), UVB (280-315 nm), and UVA (315-400 nm) as it can be seen in Figure 4.

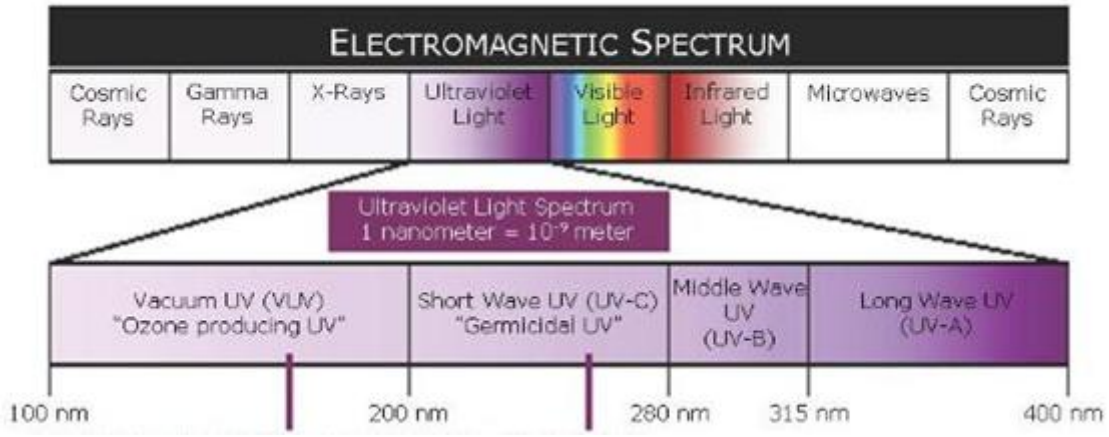

Figure 4: Electromagnetic spectrum and UV components.

The UV radiation has different biological effects; first the UV photons are transmitted through the skin layers and are absorbed by a cellular molecule. Once it occurs, the biochemical reactions take place. UV light causes damage in two different ways; one is the direct absorption of UV photons by a cellular chromophore that can lead to photo-induced reactions in DNA such as the formation cyclobutane pyrimidine bases (CPDs) [29]. The second way (indirect way) implies a photosensitization process, in which a molecule called photosensitizer absorbs UV light [30]. When the UV photons are absorbed, there is a redistribution of the electrons leading to an excited state of energy. In this state the molecule is prone to interacts with other bio-molecules transferring electrons and forming photoproducts such as free radicals; or it can transfer energy to oxygen producing reactive oxygen species (ROS) [29]. Consequently, depending on thickness of epidermis, the distribution and amount of chromophores and the undergone photobiochemical reactions; the biochemistry of the biomolecules in the skin may change.

UVC is the shortest wave and the highest energetic type, but most of it is filtered and absorbed by the ozone layer (Rayleigh scattering). Nevertheless, there are regions where the layer is thinner, contributing to UVC delivery to earth's surface [31].

UVA is the longest wave, being absorbed very little by the atmospheric ozone layer and accounting for the 95 percent of the UV radiation that reaches the earth's surface [32]. This type penetrates in deeper layers of the skin than UVB, as it can be observed in figure 5. DNA can be structurally damaged after exposure to UVA via Reactive Oxygen Species (ROS) reacting with the DNA to cause G:C to T:A guanine transversion forming 7-8, dihydro 8-oxo-guanine [33, 34, 35]. Furthermore it is reasonably recognized to be a human carcinogen and play a role in photoaging.

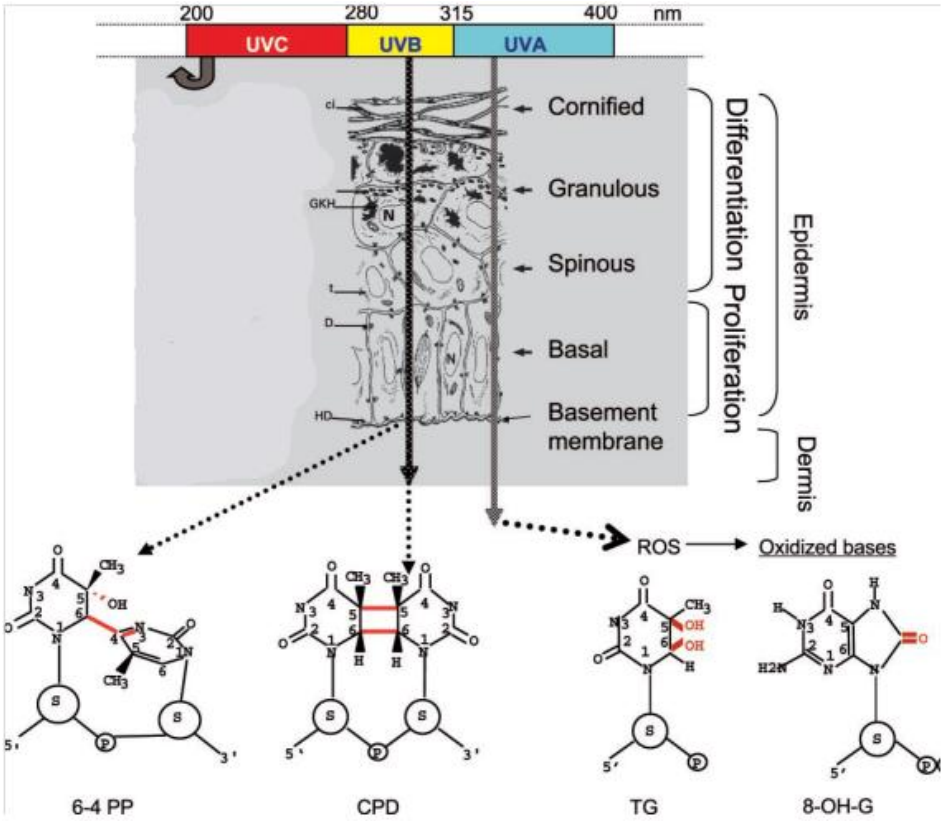

Figure 5: Scheme indicating penetration of UV types and their effects.

UVB is the middle-range in UV light emitted by Sun. It is directly absorbed at the level of the double helix in DNA giving rise to dimeric photoproducts (CC, TT, TC, CT) between adjacent pyrimidine bases and creating a bulge in DNA (figure 6). Two major types of these bulky modifications are named *cis-syn*-cyclobutane pyrimidine dimmers (CPD) and pyrimidine 6-4 pyrimidone photoadducts (6-4 PPs) and its Dewar valence isomers formed by photoisomerization of 6-4 PPs at wavelengths higher than 290 nm [32, 34, 36]. In the case of CPDs two pyrimidine bases are linked by a cyclobutane ring involving the 5 and 6 carbon atoms of both molecules and in the case of 6-4 PPs a single bond is established between the 6 carbon atom of a base and the 4 carbon atom of the other base, as it can be seen in figure 5. In such a way that the DNA

is distorted in the presence of CPD or 6-4 PP. This distortion is believed to arrange the recognition of each of the DNA modifications (CPD and 6-4 PP) by specific enzymes to repair the damage caused by UVB radiation [37]. These enzymes belong to the Nucleotide Excision Repair mechanism.

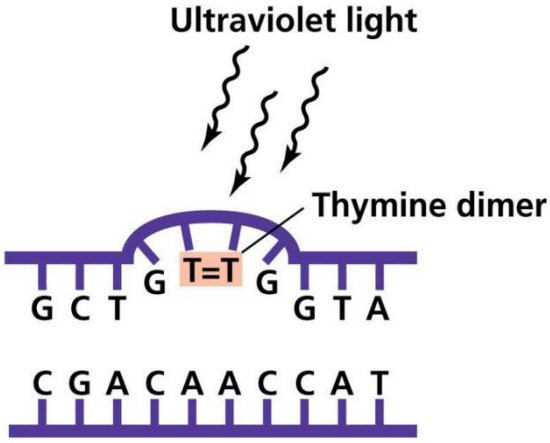

Figure 6: Bulge in DNA induced by UV

4.3.NUCLEOTIDE EXCISION REPAIR MECHANISM

Nucleotide Excision Repair is a sophisticated and highly versatile biochemical mechanism whose function is to remove the DNA lesions induced by the short wavelength but highly energetic UVB and UVC radiation, specifically those in the form of CPD and 6-4 PP. NER mechanism depends on the activity of several factors, i.e. several enzymes that participate through the different steps to repair the lesion on the DNA strands by a multiwise 'cut and patch'-type reaction [38]. Based on the recognition step in NER mechanism two modes can be distinguished: the first is called global genomic repair (GGR) and is devoted to recognize the lesion throughout the whole genome, including regions that do not undergo transcription and silent DNA [39]. The other mode is known as transcription-coupled repair (TCR); it plays the critical role of repairing lesions which affect the transcribed DNA strand of an active gene and impair the transcription activity [40].

As it can be seen in figure 7, particularly the step 1.b which concerns to the TCR mode, an elongating RNA polymerase II is arrested at the position of the lesion induced by UV, indicating that the factors involved in the initiation differ from the GGR mode [41]. In the following step 2.b of figure 7, two specific TCR-NER proteins known as Cockayne syndrome A (CSA) and B (CSB) in conjunction to XPG are believed to take the place of the stood RNA poly-II and to recruit the TFIIH to the site of the lesion.

As it is shown in the step 1.a and of figure 7, in the case of GGR mode the recognition implies the activity of XPC/HR23B complex and XPE factor; both recognize the distortion caused in the DNA by UV-induced CPDs and 6-4 PPs [42]. In the step 2.a of figure 7, two of the components of the recruited TFIIH factor called XPB and XPD open the DNA helix around the lesion and a bubble is formed.

Following in the step 3 of figure 7, the XPA protein verifies the damage and RPA allows the stabilization of the multiple proteins involved in the complex.
Next, in step 4 of figure 7; an incision of the strand in the 3' orientation is made by the XPG and the incision in the 5' orientation is undergone by ERCC1/XPF factor, resulting in a short single-strand segment of DNA and removing the lesion [43].

The fifth step of figure 7, the repair synthesis is made by DNA polymerases ε or δ using the undamaged strand as a template. In the final step of figure 7, DNA ligase completes NER process by sealing the repaired strand.

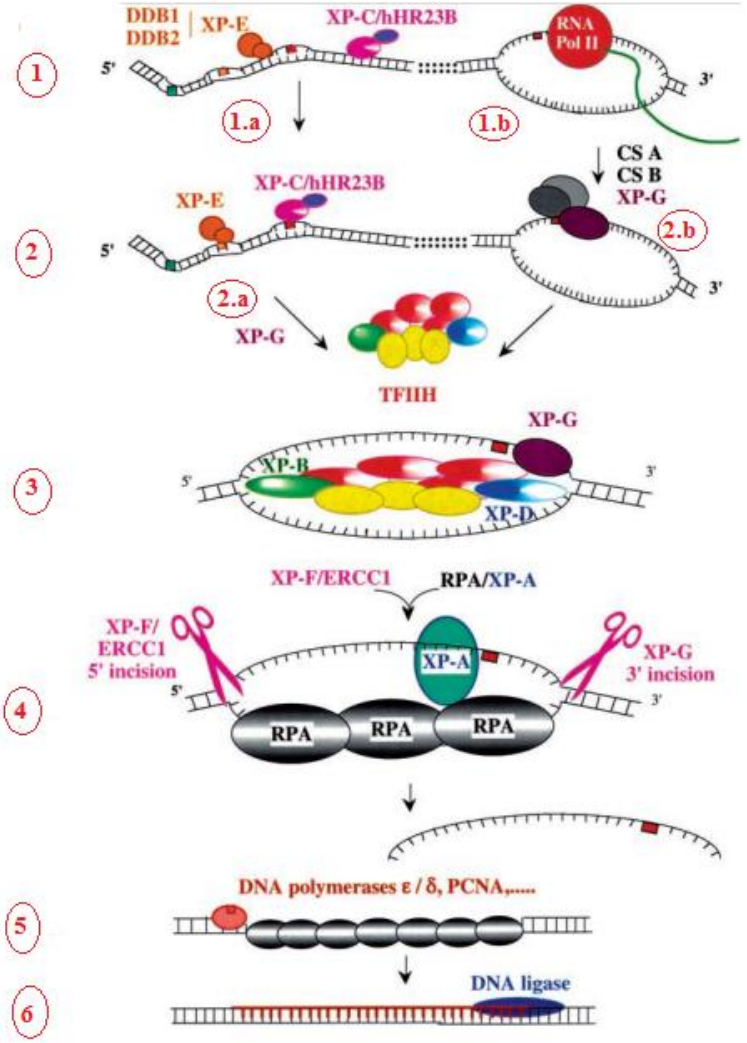

Figure 7 : Steps involving GGR and TCR modes of NER.

NER plays the critical role of maintaining the integrity of the human genome as intact as possible, since humankind is almost constantly exposed to sunlight and the mutagenic ability of its components. There are individuals are not able to repair the damage in the DNA provoked by UV by using the NER mechanism and they present Xeroderma Pigmentosum (XP).

4.4.XERODERMA PIGMENTOSUM

The term of "Xeroderma Pigmentosum" was first described by Hebra F. and Kaposi M. in 1874. In 1932 was done the first association of XP with neurological dysfunction by Sanctis and Cacchione [44]. The first link between Xeroderma Pigmentosum and the molecular basis in DNA repair deficiency was discovered in 1968 by James E. Cleaver [45].

Xeroderma Pigmentosum patients have abnormal NER mechanism, since one of the eight genes that encode for the proteins involved in the cascade is defective giving rise to the different XP types [46]. So far, there have been identified seven complementation groups, which are designated as XPA to XPG corresponding to mutations in genes XPA-XPG. The other type of XP is called XP Variant (XPV) in which there are mutations in the gene codifying for the polymerase η, which is responsible for translesion synthesis. This is "a damage-tolerance mechanism that supports the direct bypass of DNA lesions" [43].

Xeroderma Pigmentosum is a rare inherited disorder transmitted in an autosomal recessive manner, thus it affects to men and women equally and the first signs are in childhood. The usual manifestations are in small numbers worldwide with 1-5 cases per million of people taking into account all racial groups [44]. From data obtained between 1971 and 2009 the incidence of cases was approximately of 1 per 250,000 people in USA and 1 per 20,000 in Japan [36, 47]. It can be estimated 2.3 per million people in Western Europe [48]. These are general numbers, the reality may vary due to several factors such as the isolation, less mobility or places in which the people lineages are more connected as the case of Middle East, North Africa or India [36, 49, 50].

Most of the XP individuals, approximately around the 60% [49], are clinically characterized by abnormal sensitivity to sun associated to acute sunburns with blistering and persistent inflammation of the exposed areas of the skin. The rest of cases do not show sunburn reactions [48]. According to J. Lehman, at an early age most of the patients tend to develop hyper- and/or hypo-pigmentation abnormalities in the form of poikiloderma; and to have skin dryness (xerosis) as shown in Figure 8 [48, 49]. Those

abnormalities may be associated to premature skin ageing. Under the age of 10, XP individuals have high susceptibility to develop some squamous cell carcinomas, basal cell carcinomas and melanomas, because the high number of mutations these patients accumulate along their lives [37].

Figure 8: atrophic dry skin showing hyper and hypopigmentation.

It is not uncommon that in some XP patients the damage may affect the nervous system deriving in progressive conditions such as sensory-neural hearing loss, abnormal motor activity, cognitive impairment and ophthalmic manifestations. These conditions are present in patients that have the disease corresponding to XP complementation groups A, B, D, F or G [50]. In the case of XP complementation groups E and V neurologic abnormalities rarely occur [48].

4.5.XERODERMA PIGMENTOSUM COMPLEMENTATION GROUP C

XPC is the most frequent group among Caucasian population in Europe and North Africa and it is also the most prevalent worldwide, as it can be observed in Table 1.

Complementation group	Affected gene	Percentage of XP patients worldwide
XP A (MIM: 278700)	*XPA* (MIM: 611153)	9 %
XP B (MIM: 610651)	*XPB* (MIM: 133510)	1 %
XP C (MIM: 278720)	*XPC* (MIM: 278720)	43 %
XP D (MIM: 278730)	*XPD* (MIM: 126340)	28 %
XP E (MIM: 278740)	*DDB1* (MIM: 600045), *DDB2* (MIM: 600811)	3 %
XP F (MIM: 278760)	*XPF* (MIM: 133520)	< 1 %
XP G (MIM: 278780)	*XPG* (MIM: 133530)	3 %
XPV (MIM: 278750)	*Pol H* (MIM: 603968)	7 %

Table 1 : XP complementation group and prevalence

Several mutations in the XPC gene make the cells from patients with this syndrome to be unable to remove the UV-induced DNA lesions in the form of CPDs and 6-4 PPs [36]. It has been demonstrated that only the Global Genomic-NER is affected in XPC individuals [39, 51]. It is clear that all the cells need from XPC protein activity to recognize the damage in the DNA; nevertheless whether it has been synthesized from the mutated gene or even though it is absent, its damage detection will not be performed. Consequently the recruitment of the following factors will not occur so the GG-NER will not be initiated [40]. This leads to a low repair capacity of the Nucleotide Excision Repair (NER) mechanism, since the other mode still works [41]. These unrepaired lesions are accumulated throughout the genome of cells resulting in cellular death by apoptosis or maybe causing DNA replication errors which lead to mutations [43].

The common cutaneous abnormalities of the XP group C are freckle-like pigmentation in those skin areas exposed to the skin, this tend to be alternated with hypopigmentation, telangiectasia, and atrophy [52]. They are prone to develop multiple skin carcers; including squamous and basal cell carcinomas and melanomas [39, 51,52]. In addition, XPC patients rarely develop neurological manifestations [50].

4.6.P53 AND Ki-67

The tumor suppressor gene p53 encodes for the protein p53, which is involved in the regulation of some processes that directly affect the cell since this protein mediates in the cell cycle. This protein is in a deactivated form, until the cell is stimulated by intracellular or extracellular stimuli, including a lesion in the DNA, oncogene over-expression, hypoxia when there is lack of oxygen, or a heat shock. In the particular case, when UV light induces damage to the DNA in the form of CPDs or 6-4 PPs, p53 is activated. Then the cell may follow two possible options; in the first one the cell cycle is stopped, the cell growth is interrupted and the cell is allowed to repair the DNA damage. If the damage is repaired the cell cycle will restart. In the case that the damage is very severe, the cell will follow the second option, i.e. the programmed cell death called apoptosis, as it is illustrated in figure 9. In this way, the genome integrity is maintained.

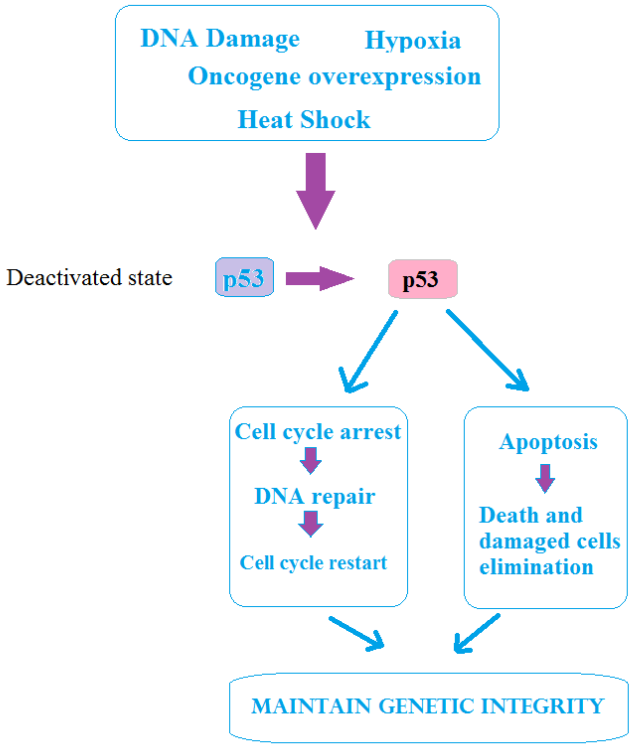

Figure 9: p53 pathways.

The Ki-67 gene encodes for the protein Ki-67, which is strictly associated to cell growth or proliferation. It is present in the active phases of the cell cycle, i.e. when the cell is growing and dividing. This makes it an excellent marker to determine how much or not a cell population proliferates [53].

5.MATERIALS AND METHODS

Primary fibroblasts and keratinocytes from healthy donors and Xeroderma Pigmentosum complementation group C (XPC) patient were obtained from unexposed skin sites through skin biopsies and kindly provided by Dr. Nagore.

CULTURE MEDIA

The cells were sown over sterilized crystals to get high amount of cells in a limited area and placed in the culture plates.

On the one hand, dermal fibroblasts were cultured in a Dulbecco's modified Eagle (DMEM (IX) + GlutaMax) medium containing 1% of antibiotic (Penicillin-Streptomycin) and 10% of Fetal Bovine Serum (FBS).

On the other hand, keratinocytes were cultured over a feeder layer. The medium of keratinocytes was made by Dulbecco´s modified Eagle medium containing Ham's F12 medium (F12 Nut Mix 1X + Glutamax), 10% fetal bovine serum, 1.3 ng/ml of triiodothyronine (T_3), 5 mg/ml of insulin, 10 ng/ml of epidermal growth factor (EGF), 24 mg/ml of adenine, 0.4 mg/ml hydrocortisone, 8 ng/ml cholera enterotoxin and antibiotic/ antimicotic 1% keratinocyte medium (KCA).
The fibroblasts were used to perform the photosensitivity assay. Fibroblasts were grown until the necessary confluence is reached and then they were irradiated to perform the experiment.

The keratinocytes were used to perform an adhesion study. This is a method of 3D culture system, in which the integrin-mediated adhesion of the cells to the matrix is improved since there is more contact surface. The keratinocytes were grown in a mixed medium containing KCA and Cnt-57 (1:1) which is a progenitor cell targeted liquid culture medium ('CellNTec') [54] and placed over three different scaffolds.

The cultured fibroblasts and keratinocytes were grown in an incubator at 37º in which the atmosphere was humidified with 5% of CO_2.

MATRIX PREPARATION

Three different matrices of were used: collagen, matrigel and fibrin:

Collagen type I is one of the most used extracellular matrix proteins for cell culture systems (figure 10.A). It is used to promote cell adhesion and/or cell proliferation [55]. The collagen type I matrix was prepared with collagen solution diluted at 50 µg/ml in KCA medium.

Matrigel is a gel-like substance (figure 10.B) composed of a mixture of proteins and derived from tumor cells of mice [56]. The Matrigel matrix was prepared in 1:15 proportion of matrigel solution itself with keratinocytes medium in the case of immunofluorescence studies.

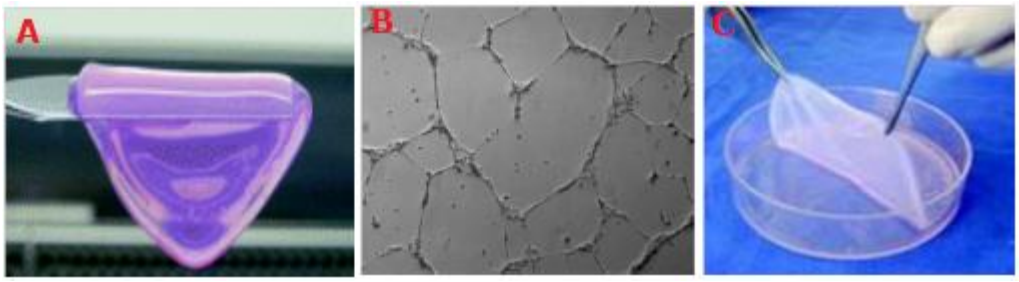

Figure 10: A) Collagen I matrix; B) Matrigel matrix; C) Fibrin matrix.

Fibrin is the principal structural component of blood clots, where it serves as scaffold to promote tissue repair; and it also has a role in the formation of blood vessels [57]. Fibrin matrix is based on the cleavage of its precursor called fibrinogen [58]. Briefly, 1 ml of fibrinogen (from blood cryoprecipitates) was added to 2 ml of DMEM with 10% fetal calf serum, followed immediately by the addition of 250 ml of 0.025 mM $CaCl_2$ (Sigma, St Louis, MO) containing 11 IU of human thrombin (Sigma). This mixture was allowed to clot at 37 °C in 5 mm tissue culture well plates (figure 10.C). After 24 hours, healthy human and XPC keratinocytes were sown over sterilized crystals and allowed to grow submerged until reaching confluence.

IRRADIATION METHODOLOGY

The UV radiation device used in the experiment was Philips, UVB TL20W/12RS (figure 8); which just emitted radiation in the range of UV type B.

Figure 11: UV lamp

Before developing the experiment, previous studies were needed to determine the appropriate dose of radiation. In this way, fibroblasts were exposed to different radiation doses, particularly 200 mJ/cm^2, 400 mJ/cm^2 and 600 mJ/cm^2. After performing immunofluorescence studies, it was reached to the conclusion that those doses were not enough to UVB induced the desired cytotoxic effect in fibroblasts. Finally, it was decided that fibroblasts were irradiated using a higher dose corresponding to 700 mJ/cm^2. After UVB radiation, fibroblasts were placed at the incubator at 37ºC until they were fixed.

CELL FIXATION

The irradiated fibroblasts were incubated and blocked at different time periods: 2h, 24h, 48h and 72h after irradiation; to observe the UV-induced DNA damage in the form of CPDs and the posterior repair process of both healthy and XPC fibroblasts. As control, no irradiated healthy and XPC cells were used to guide the studies. All the fibroblasts were fixed first with 4% formalin in PBS (e.g. a buffer solution called phosphate buffered saline) at room temperature and finally permeabilized with 0.5% of Triton X-100 in PBS at 4ºC

In the case of the case of adhesion study, once the keratinocytes were grown over the collagen, fibrin and matrigel scaffolds, they were fixed first with 4% formalin in TBS at room temperature and afterwards they were permeabilized using 0.5% of Triton X-100 in TBS at 4ºC.

IMMUNOFLUORESCENCE

The inmunofluorescence assay is one of the most common application of antibody conjugation. It consists in tagging antibodies with fluorochromes (fluorophores) making the antibodies visible under a fluorescence microscope. The use of antibodies conjugated with different fluorochromes allows the detection of different antigens at the same time when they have different specimen origin [59].
Two antibodies were used to imnunolabel the sample, they are called primary and second antibody. The primary antibody which was unlabeled reacted with the antigen, meanwhile the secondary labeled antibodies attached to their corresponding first antibodies (figure 12). Additionally the secondary antibody can be labeled with a fluorescent dye that in the two performed studies correspond to Fluorescein isothiocyanate (FITC) (green fluorescence) and Texas Red (TR) (red fluorescence).

Figure 12: Scheme of the antibody binding

On the one hand, three different biological markers were object of the study, cyclobutane pyrimidine dimers (CPDs), p53 and proliferation. First DNA of the cells was denatured by immersing them in 2M HCl during 30 min. Secondly 20% of FBS in PBS was used to prevent non-specific antibody binding. Then they were incubated during 1-2 hours with their corresponding antibody: CPD (1:1000 in 20% FBS in PBS), p53 (1:50 in 20 % FBS in PBS) and Ki67 (ready to use). Then the cells were washed several times and they were incubated during 45 minutes with their corresponding secondary antibody: CPDs and p53 (anti-mouse FITC at a dilution of 1:100 in 20% FBS in PBS) and Ki67 (anti-rabbit at a dilution of 1:100 in 20% FBS in PBS).

On the other hand, two biological markers were the object of the study, i.e. the adhesion proteins that allowed the attachment of both the normal human and XPC keratinocytes to the different matrices: alpha 6 and beta 1 integrins. First, 1% FBS in TBS (e.g. tris buffered saline) was used to prevent non-specific antibody binding. Then, the cells were incubated with their corresponding antibody during 1-2 hours: alpha 6 (1:50 in 1% FBS in TBS) and beta 1 (1:50 in 1% FBS in TBS). Then the cells were washed several times and they were incubated during 45 minutes with their

corresponding secondary antibody: alpha 6 (anti-rat FITC at a dilution of 1:250 in 1% FBS in TBS) and beta 1 (monoclonal anti-mouse Texas Red (TR) at a dilution of 1:250 in 1% FBS in TBS).

Finally, another fluorescent staining called DAPI was used to label the nuclei of the cells.

6.RESULTS AND DISCUSSION

In this section all the result obtained will be deeply exposed. The first part is corresponded to the study of the sensitivity that XPC cells have to the UVB, i.e. photosensitivity; whereas the second is devoted to the results of the adhesion study. All the cells were analyzed using the immufluorescence technique, as previously mentioned.

6.1.PHOTOSENSITIVITY STUDY

This assay was developed by using a controlled dose of UVB, as mentioned in section (materials and methods) it corresponds to 700 mJ/cm^2, to examine the cytotoxic effect induced in XPC skin fibroblasts, the main type of dermal cells. The damage induced by UVB will be studied by analyzing the presence of CPDs in the both healthy and XPC fibroblasts. The same number of cells was sown throughout the whole photosensitivity approach.

As it can be seen in figure 13.A and 13.C, the levels of cyclobutane pyrimidine dimmers (CPDs) were negative in both healthy and XPC fibroblasts, respectively; since they have not been irradiated and UVB has not been able to induce damage to the DNA. The figure 13.B and 13.D illustrate the presence of cells nuclei labeled with DAPI.

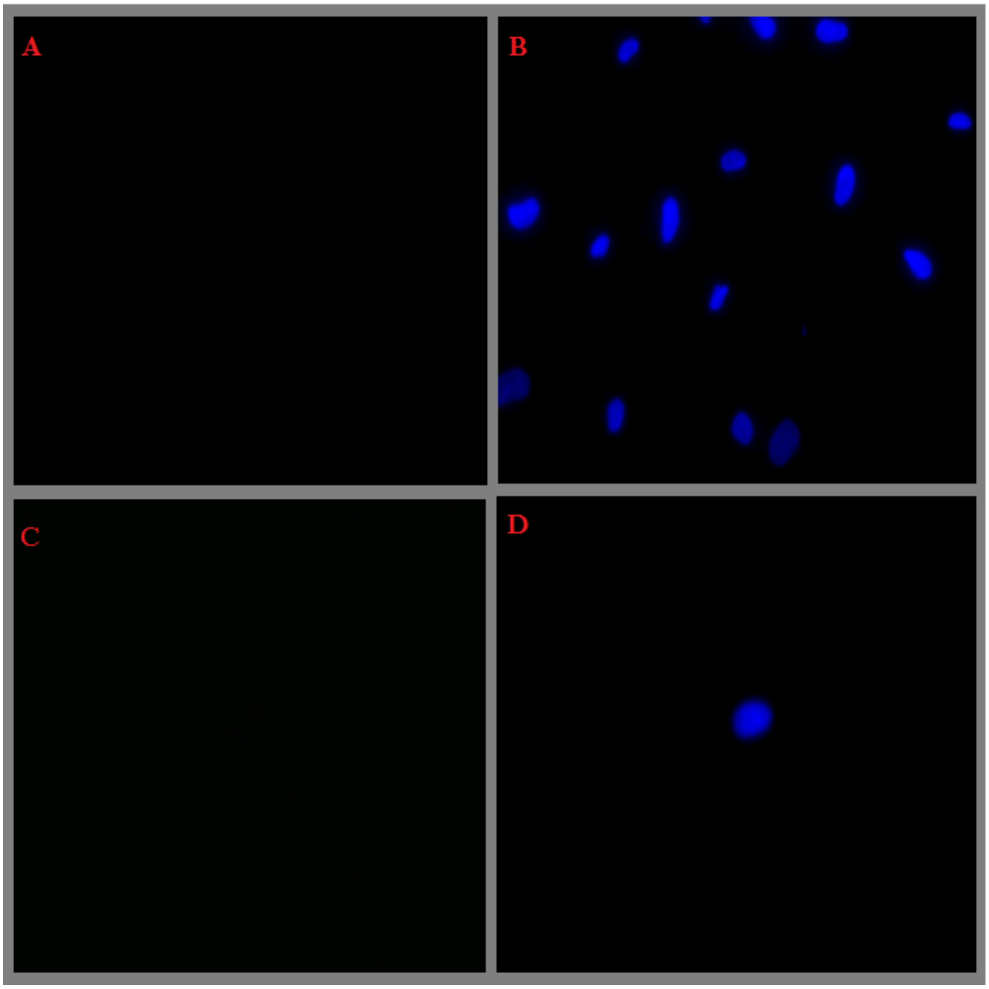

Figure 13: Cells without radiation: A) HHF dimmers FITC; B) HHF dimmers DAPI; C) XPC dimmers FITC; D) XPC dimmers DAPI

As it is shown in figure 14.A and 14.C in healthy and XPC cells, levels of p53 protein are negative because the UVB has not induced any kind of damage to the DNA of the cell; then p53 is not expressed. This result is as expected and it is also described by Bernerd F. et al, that in the absence of UV-B, p53 is also absent [2]. DAPI also indicates the presence of the nuclei of cells, as it is shown in figure 14.B and 14.D.

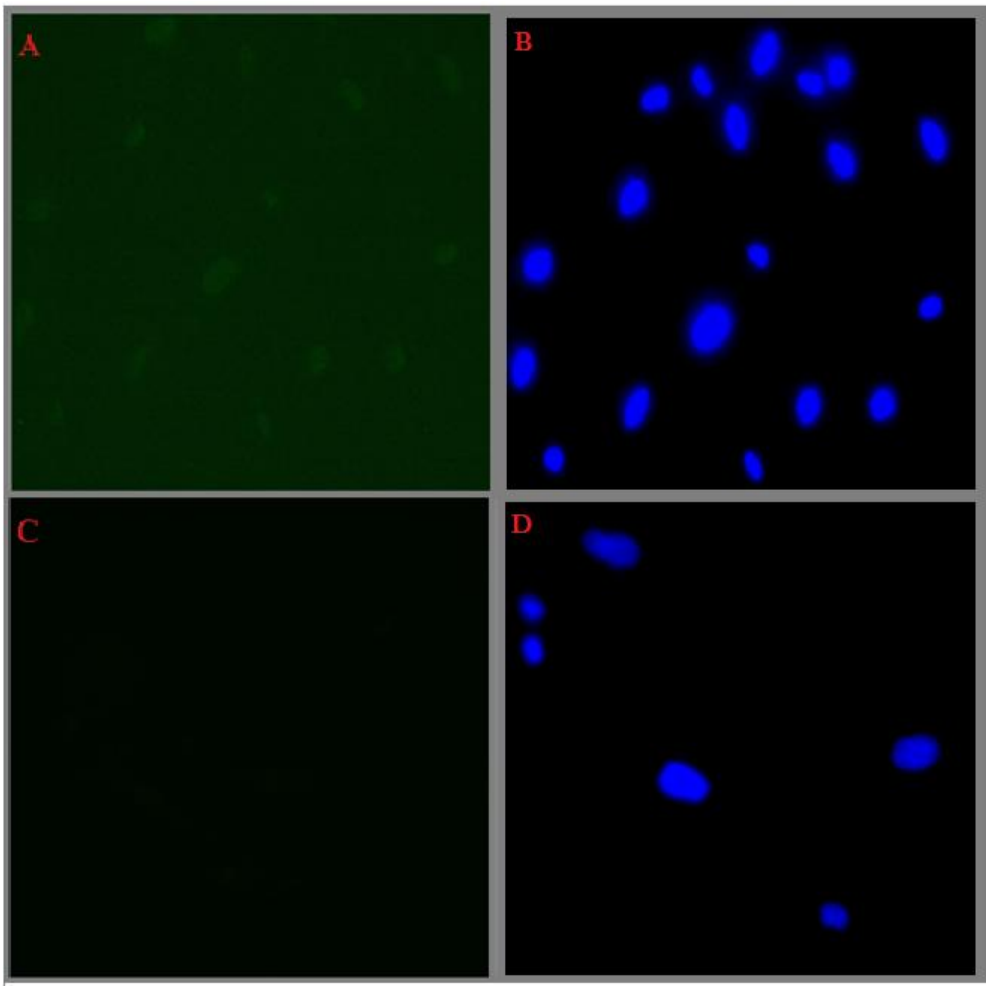

Figure 14: Cells without radiation: A) HHF p53 FITC; B) HHF p53 DAPI; C) XPC p53 FITC; D) XPC p53 DAPI

The figures 15.A and 15.C illustrate positive levels of the protein Ki-67. This coincides with the stated in section 4 (introduction) that this protein is present in the active phases of the cell cycle. Therefore, this suggests that a relation can be obtained between the deactivated state of p53 protein (negative levels in figures 14.A and 14.C) and the positive levels of Ki-67 indicating that healthy and XPC fibroblasts proliferate as usually do because the cell cycle has not been interrupted. DAPI staining verifies the presence of nuclei of both healthy and XPC fibroblasts in figure 15.B and 15.D.

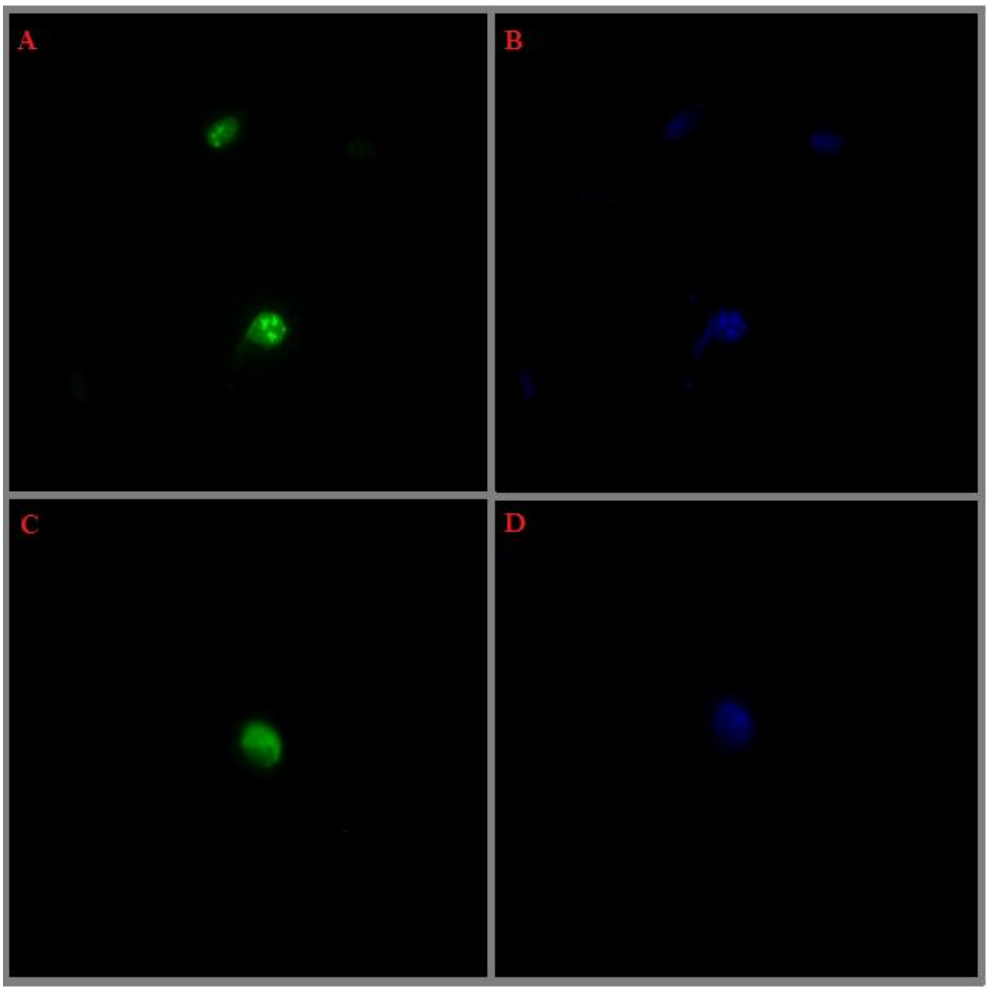

Figure 15: Cells without irradiation: A) HHF KI-67 FITC; B) HHF KI-67 DAPI; C) XPC KI-67 FITC; D) XPC KI-67 DAPI

The figures 16 (A, B, C, D, E and F) show the cells after 2 hours of being irradiated. First, healthy and XPC fibroblasts show positive levels of CPDs, as indicated in figure 16.A and 16.D respectively. Positive levels of p53 protein are shown in figures 16.B and 16.E corresponding to healthy and XPC cells respectively. At this time, UVB has already induced damage into DNA, the cells have detected it and they express p53 protein. P53 is supposed to stop the cell cycle, and in this way the proliferation is also stopped; as it is illustrated in the negative levels of Ki-67 in figures 16.C and 16.F of healthy and XPC fibroblasts, respectively. Thus, these results are in agreement with what was expected.

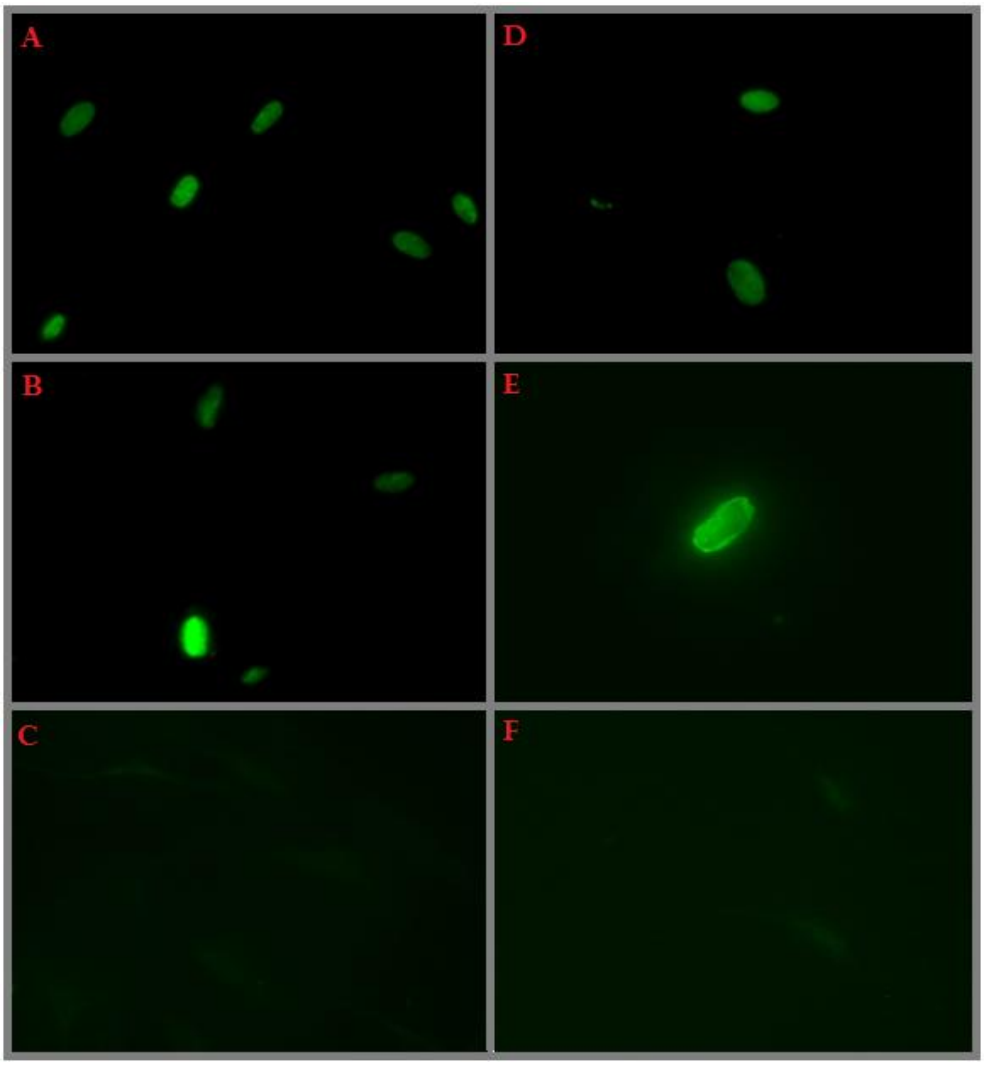

Figure 16: Cells after 2H of radiation: A) HHF DIMMERS FITC; B) HHF p53 FITC; C) HHF KI67 FITC; D) XPC DIMMERS FITC; E) XPC p53 FITC; F) XPC KI67 FITC

The figures 17 (A, B, C and D) illustrate the cells after 24 hours of being irradiated. The levels of CPDs (figure 17.A and 17.B) are also positive as in the case of the radiation after 2 hours (figures 16.A and 16.D) in both healthy and XPC fibroblasts, respectively. DAPI staining reveals the presence of nuclei of both healthy and XPC fibroblasts in figure 17.B and 17.D.

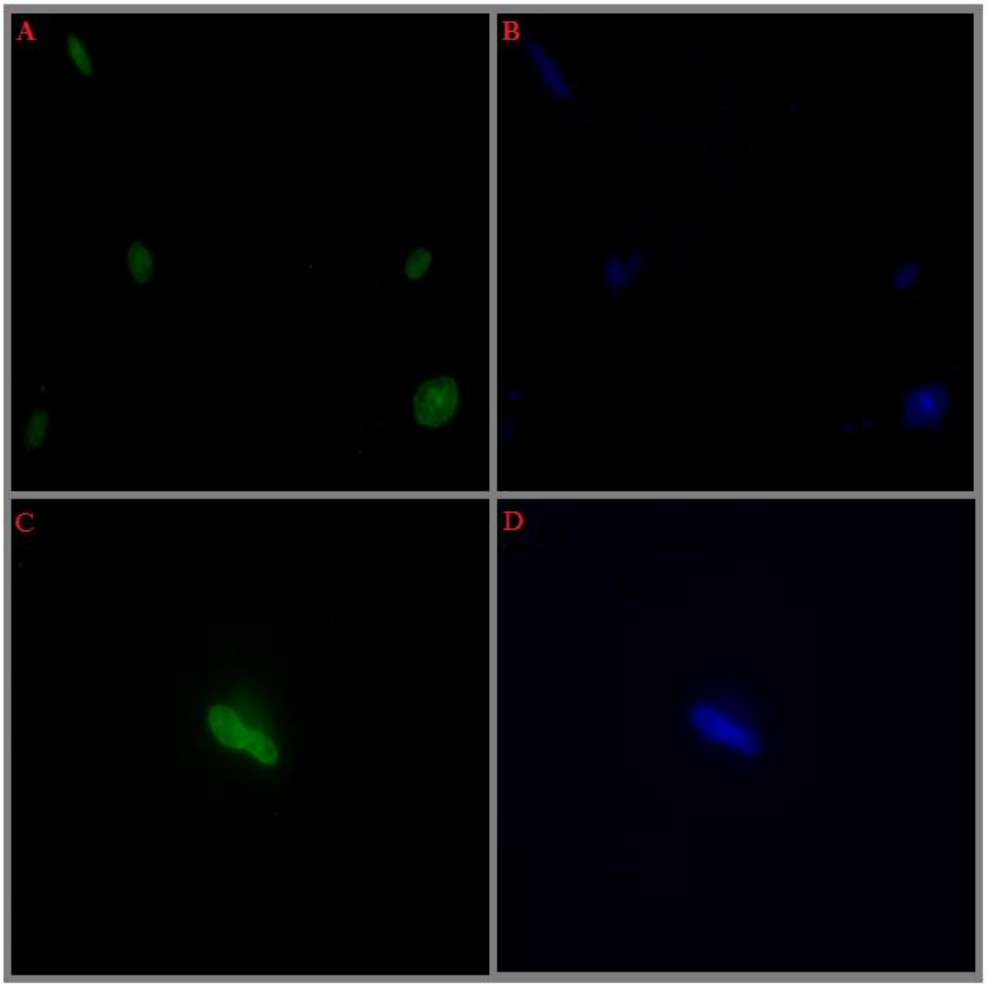

Figure 17; Cells after 24 hours of radiation: A) HHF DIMMERS FITC; B) HHF DIMMERS DAPI; C) XPC DIMMERS FITC; D) XPC DIMMERS DAPI

In the figures 18 (A, B, C and D) are shown the healthy and XPC after 24 hours of radiation, illustrating the p53 levels. As it can be seen, in figures 18.A and 18.B the levels of p53 protein are positive, the same that happens after 2 hours of radiation (figure 16.B and 16.E) in both healthy and XPC fibroblasts, respectively.

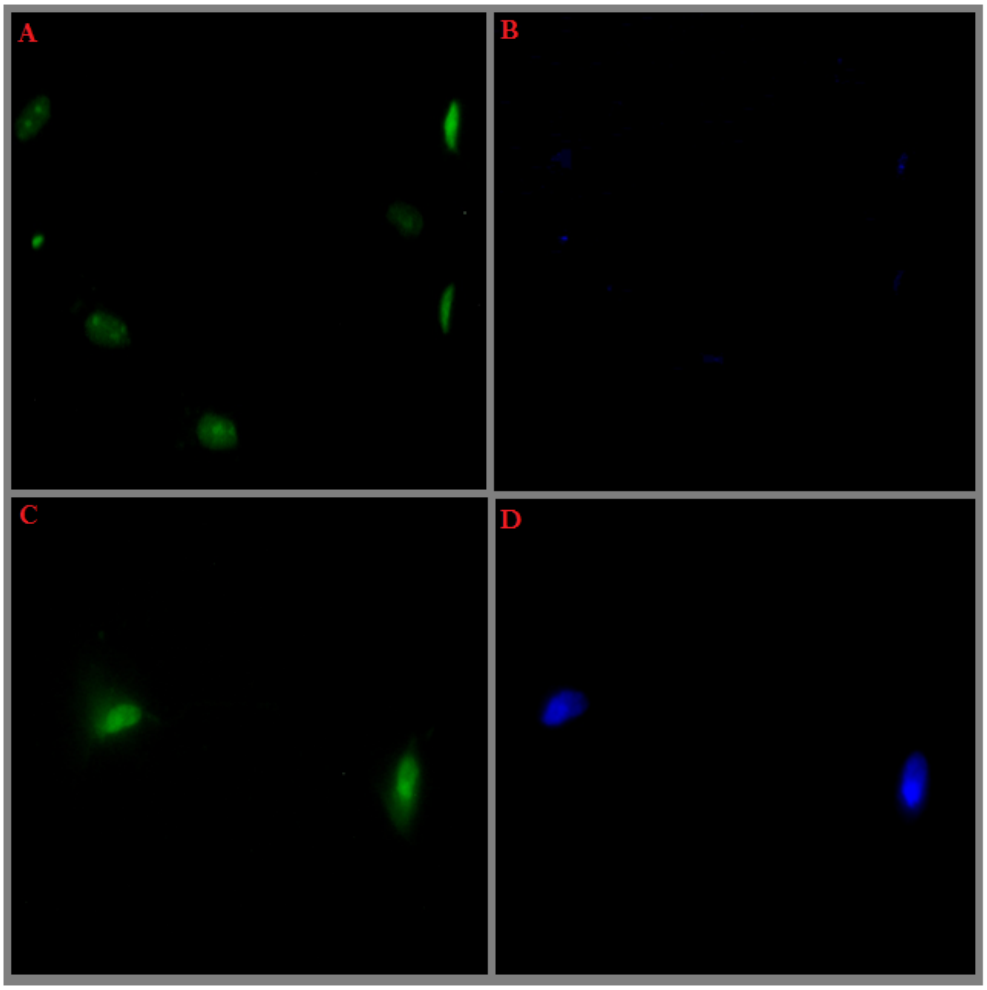

Figure 18; Cells after 24 hours of radiation: A) HHF p53 FITC; B) HHF p53 DAPI; C) XPC p53 FITC; D) XPC p53 DAPI. In B and D nuclei are stained with DAPI

The figures 19 (A, B, C and D) show the levels of expression of Ki-67 after 24 hours of radiation of the cells. Ki-67 the levels are also negative as indicated in figures 19.A and 19.B; this occurs in an equal manner in the previous figures 16.C and 16.F in both healthy and XPC fibroblasts. DAPI staining clearly reveals the presence of nuclei of both healthy and XPC fibroblasts, as indicated in figures 19.B and 19.D respectively.

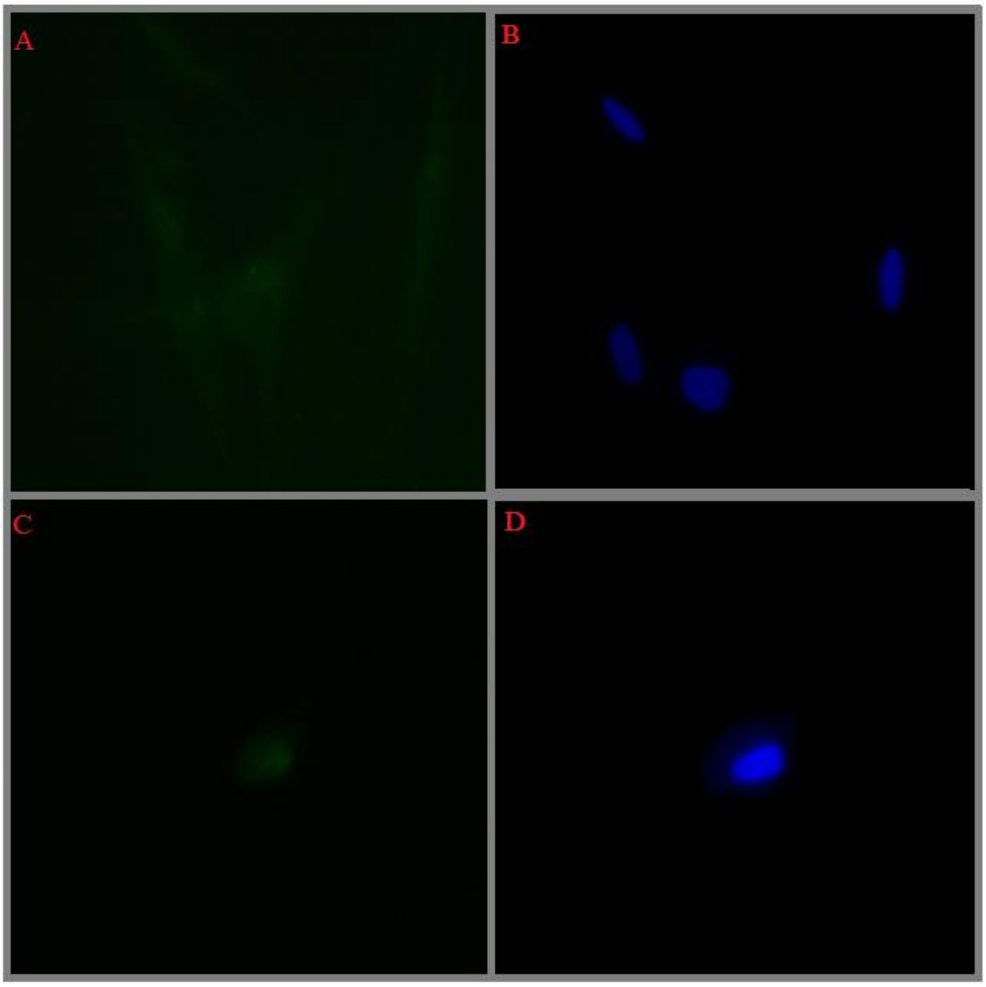

Figure 19; Cells after 24 hours of radiation: A) HHF KI67 FITC; B) HHF KI67 DAPI; C) XPC KI67 FITC; D) XPC KI67 DAPI. In B and D nuclei are stained with DAPI.

In the figures 20 (A, B, C, D, E and F) are shown the response of cells after 48 hours of irradiation. First, it can be observed a difference in the immunolabeling of CPDs in the examined fibroblasts; i.e. levels of dimmers in healthy cells (figure 20.A) are clearly lower than in the case XPC cells (figure 20.D) which remain positive as in the previous exposed cases (see figures 16.D and 17.C). This manifests the capacity of healthy fibroblasts to repair the UVB-induced dimmers in the DNA by using NER mechanism.

Conversely, the XPC fibroblasts are not able to repair this damage; since, as mentioned in the introduction, the gene encoding the XPC protein is defective and the global genomic NER mechanism cannot be undergone.

The same difference can be seen in the case of p53 protein; which in healthy fibroblasts is almost no expressed (figure 20.B), in contrast to XPC cells (figure 20.E) in which this protein continues being expressed due to the DNA damage has not been repaired, as in previous described cases (see figures 16.E and 18.C).

Respect to Ki-67, its levels start to be positive in healthy cells (figure 20.C) and continue being negative in XPC fibroblasts (figure 20.F). This suggests that since XPC fibroblasts are not able to repair DNA damage using the GG-NER, p53 is expressed because it is detected damage in the DNA and this protein continues giving the chance to repair dimmers.

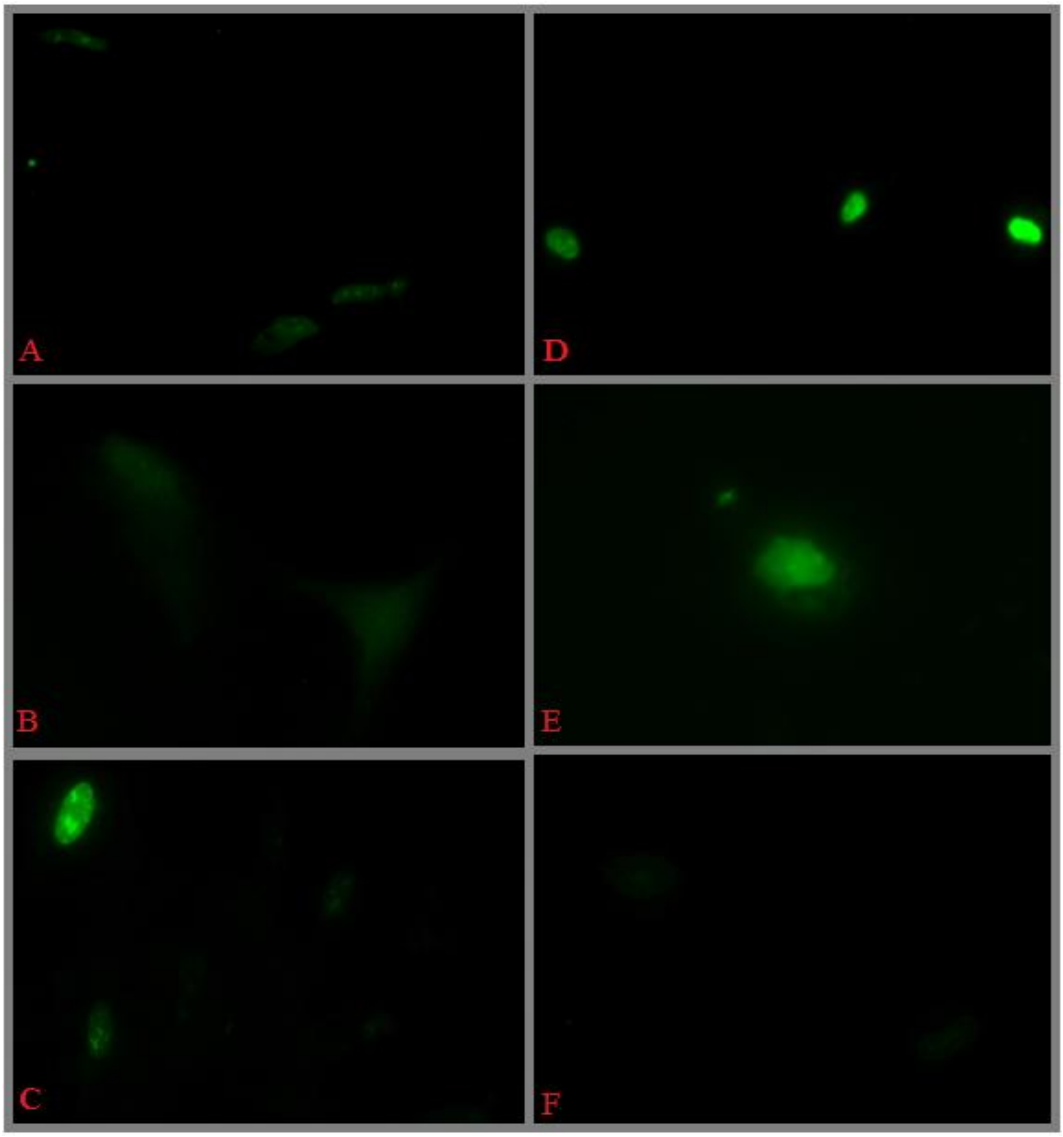

Figure 20: Cells after 48H of radiation: A) HHF DIMMERS FITC; B) HHF p53 FITC; C) HHF KI67 FITC; D) XPC DIMMERS FITC; E) XPC p53 FITC; F) XPC KI67 FITC

The figure 21 shows the response of the fibroblasts 72 hours after the radiation. At this time, healthy fibroblasts (figure 21.A) have completely recovered from the UVB-induced damage showing negative levels of CPDs. Meanwhile, XPC fibroblasts maintain the same levels of dimmers in their DNA, as it is illustrated in figure 21.C.

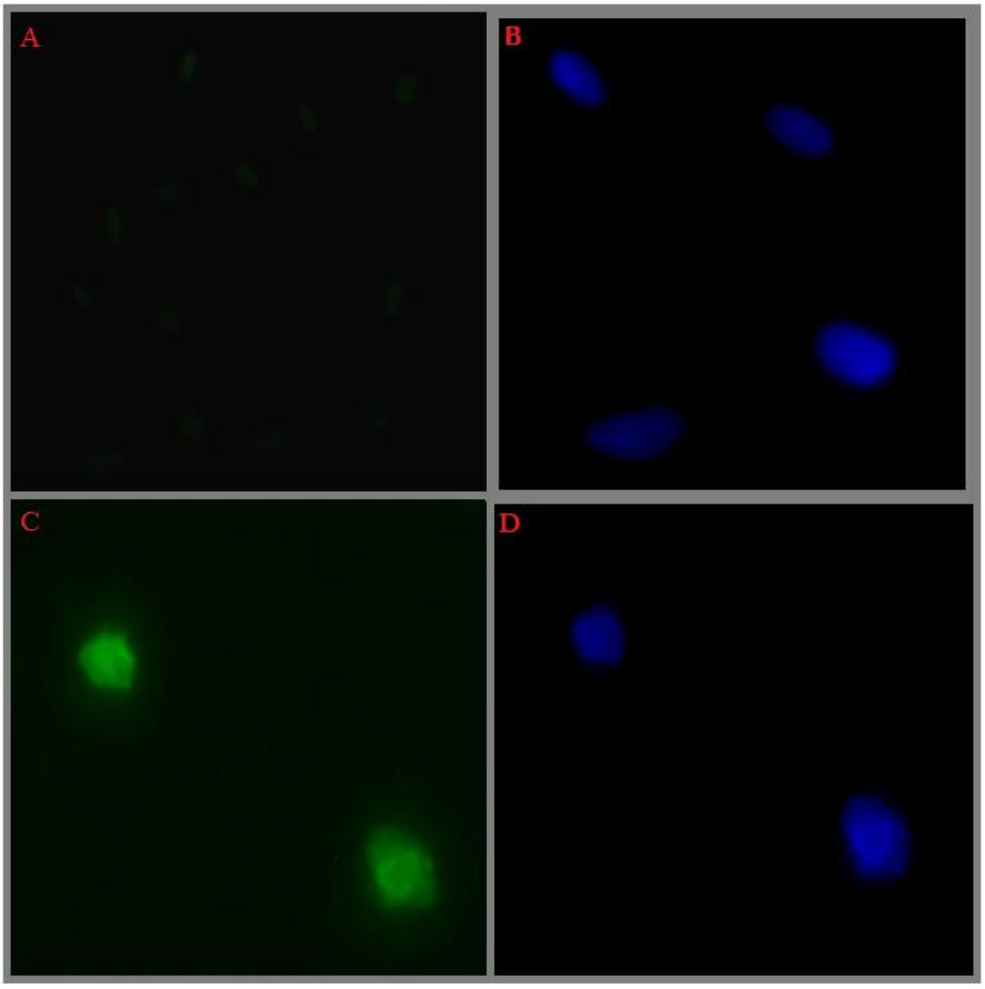

Figure 21; Cells after 72 hours of radiation: A) HHF DIMMERS FITC; B) HHF DIMMERS DAPI; C) XPC DIMMERS FITC; D) XPC DIMMERS DAPI. In B and D nuclei are stained with DAPI

As previously described in 48 hours, at 72 hours the same pattern is followed. As it can be observed in figure 22. In this line, p53 protein is completely unexpressed in healthy fibroblasts (figure 22.A); conversely, XPC cells continue expressing it (figure 22.C).

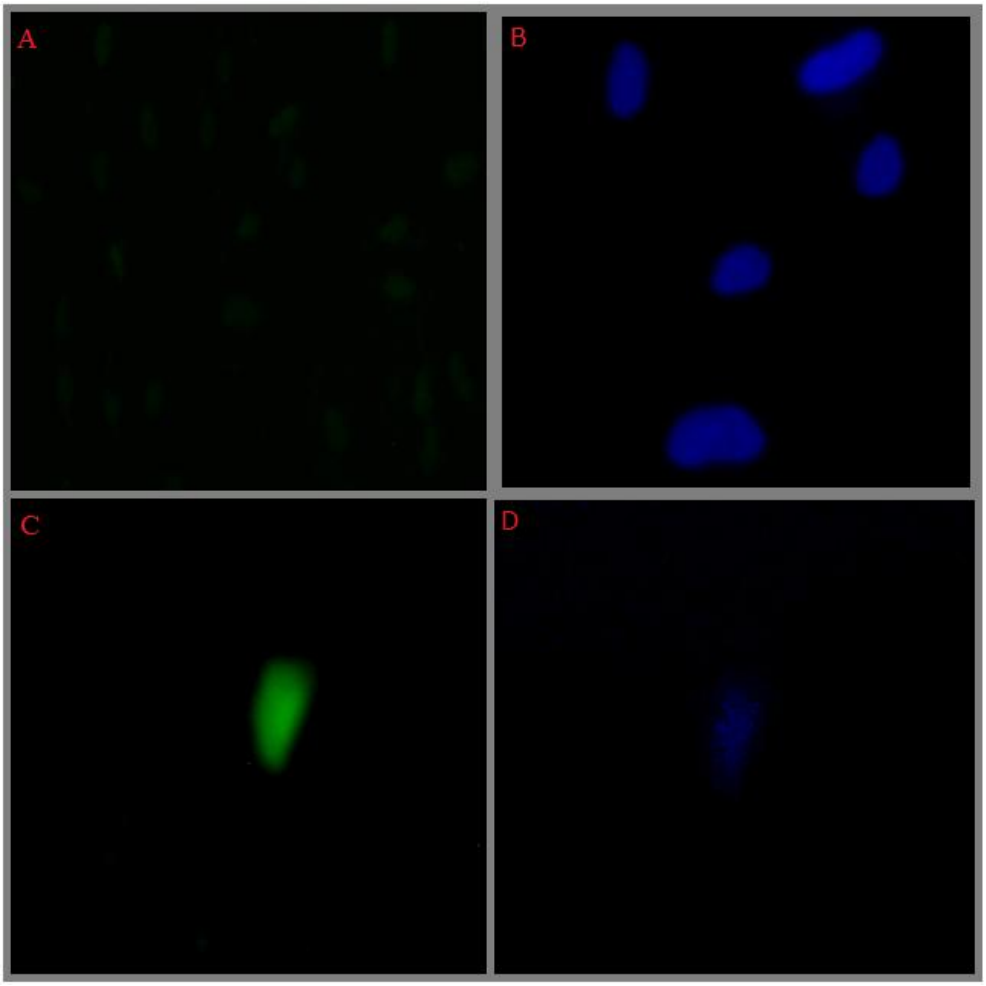

Figure 22; Cells after 72 hours of radiation: A) HHF p53 FITC; B) HHF p53 DAPI; C) XPC p53 FITC; D) XPC p53 DAPI. In B and D nuclei are stained with DAPI.

However clearly difference can be observed in the expression of Ki-67 levels after 72 hours of irradiation. On the one hand, healthy fibroblasts have positive levels (figure 23.A) of Ki-67 indicating that they have recovered from the DNA lesions and suggesting that the cell cycle is completely reestablished. On the other hand, XPC fibroblasts present negative levels of Ki-67 expression (figure 23.C).

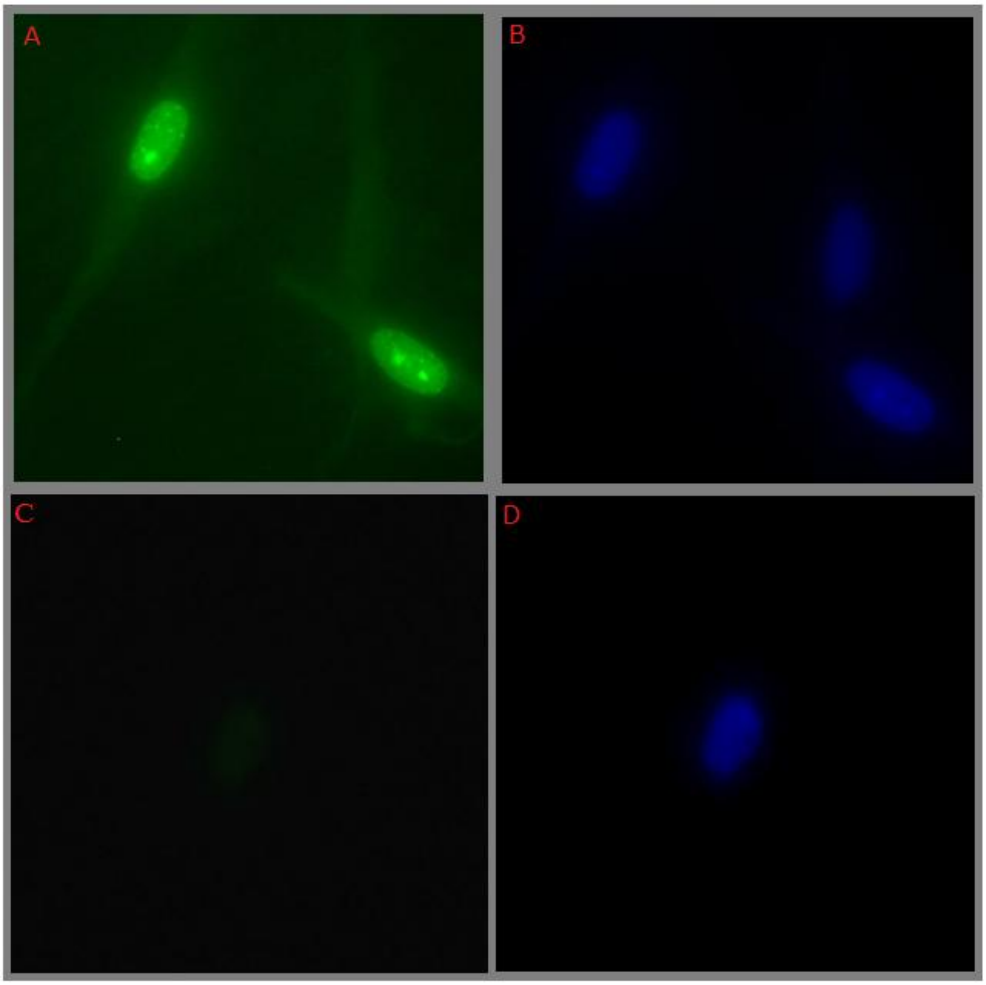

Figure 23; Cells after 72 hours of radiation: A) HHF KI67 FITC; B) HHF KI67 DAPI; C) XPC KI67 FITC; D) XPC KI67 DAPI

6.2. ADHESION STUDY

The study was developed using three different matrices: collagen, fibrin and matrigel. Healthy human keratinocytes were used as control and XPC keratinocytes were the object of the experiment. This was done to find the best matrix as a scaffold to a possible treatment. Because the integrin-mediated attachement to the basement membrane plays an active role in cell proliferation, the ß1 and α6 integrin subunits were analyzed using the immunofluorescence technique.

As it is shown in bright field in figure 24, the three matrices (collagen I, fibrin and matrigel) are viable for the growth of this cell type.

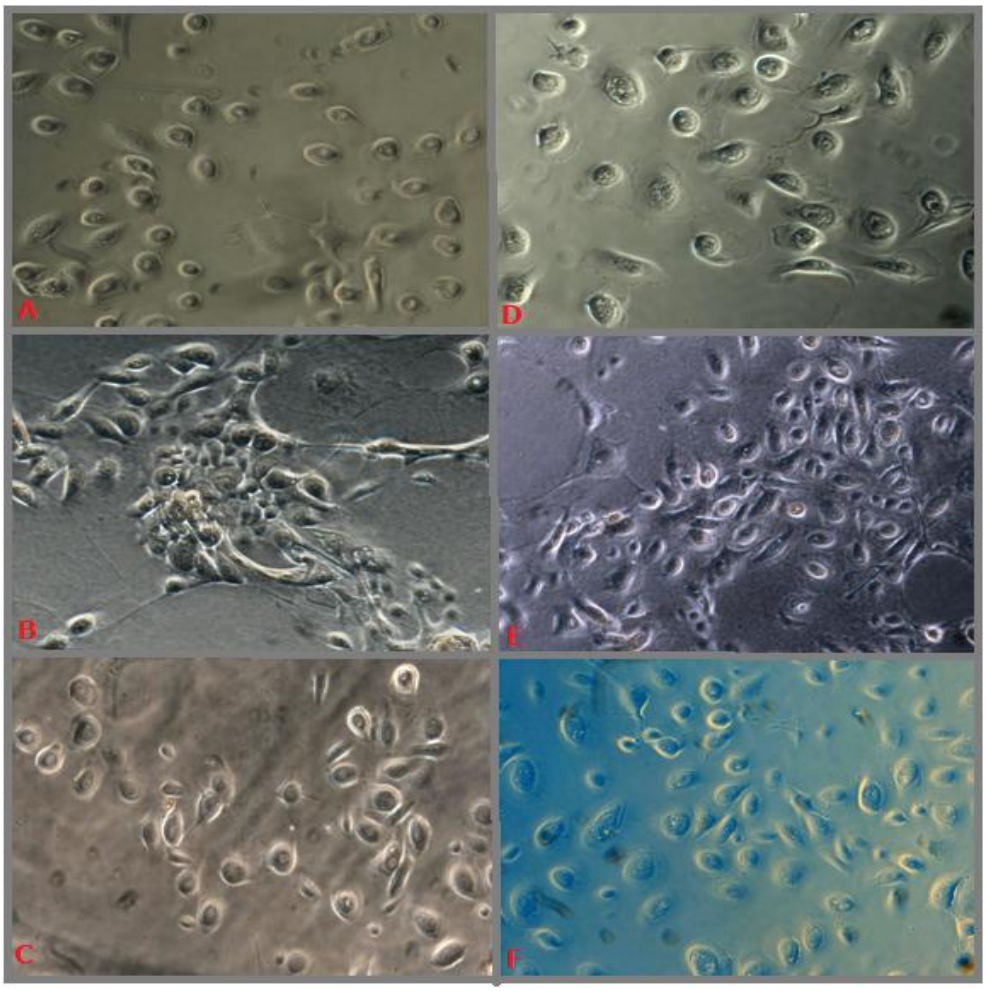

Figure 24: A) Healthy keratinocytes growing over collagen; B) XPC keratinocytes growing over collagen; C) healthy keratinocytes growing over fibrin; D) XPC keratinocytes growing over fibrin; E) healthy keratinocytes growing over matrigel; F) XPC keratinocytes growing over matrigel

ADHESION OVER COLLAGEN

It can be observed that the ß1 integrin subunit in both healthy and XPC kerartinocytes is not expressed (figures 25.A and 25.E respectively). In the case of the α6 integrin subunit; it is no expressed in neither healthy nor XPC keratinocytes, (figures 25.C and 25.G respectively). In figures 25 (B, D,F,H) DAPI stains the nuclei of the cells.

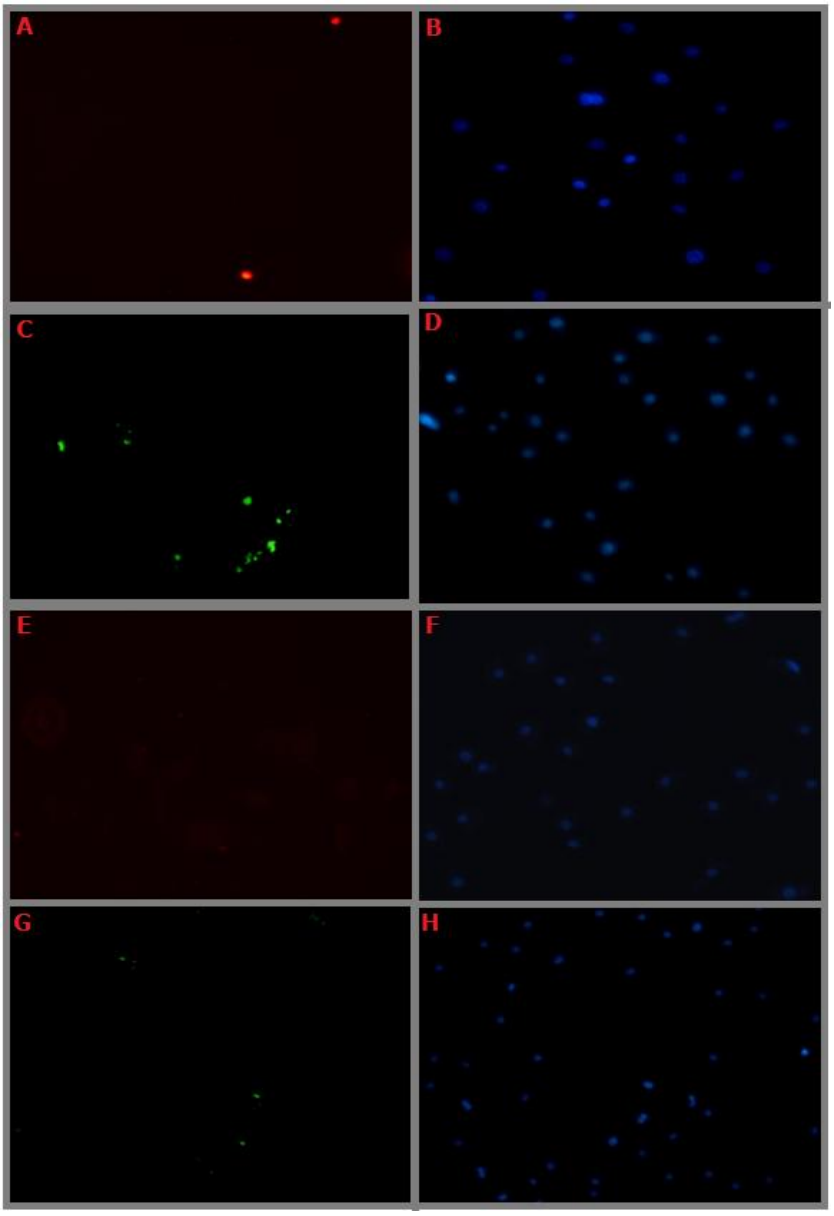

Figure 25. Cells over collagen I matrix: A) HK ß1 in collagen TR;B) HK ß1 in collagen DAPI, C) HK α6 in collagen FITC; D) HK α6 in collagen DAPI; E) XPC ß1 in collagen TR; F) XPC ß1 in collagen DAPI; G) XPC α6 in collagen FITC; H) XPC α6 in collagen DAPI

ADHESION OVER FIBRIN

It can observed that the expression of ß1 integrin subunit is negative in both healthy and XPC keratinocytes, figures 26.A and 26.B respectively. As it is illustrated in figure 26.C there is a low expression of the α6 integrin subunit corresponding to healthy keratinocytes; conversely in the case of XPC keratinocytes (figure 26.D) the expression is negative.

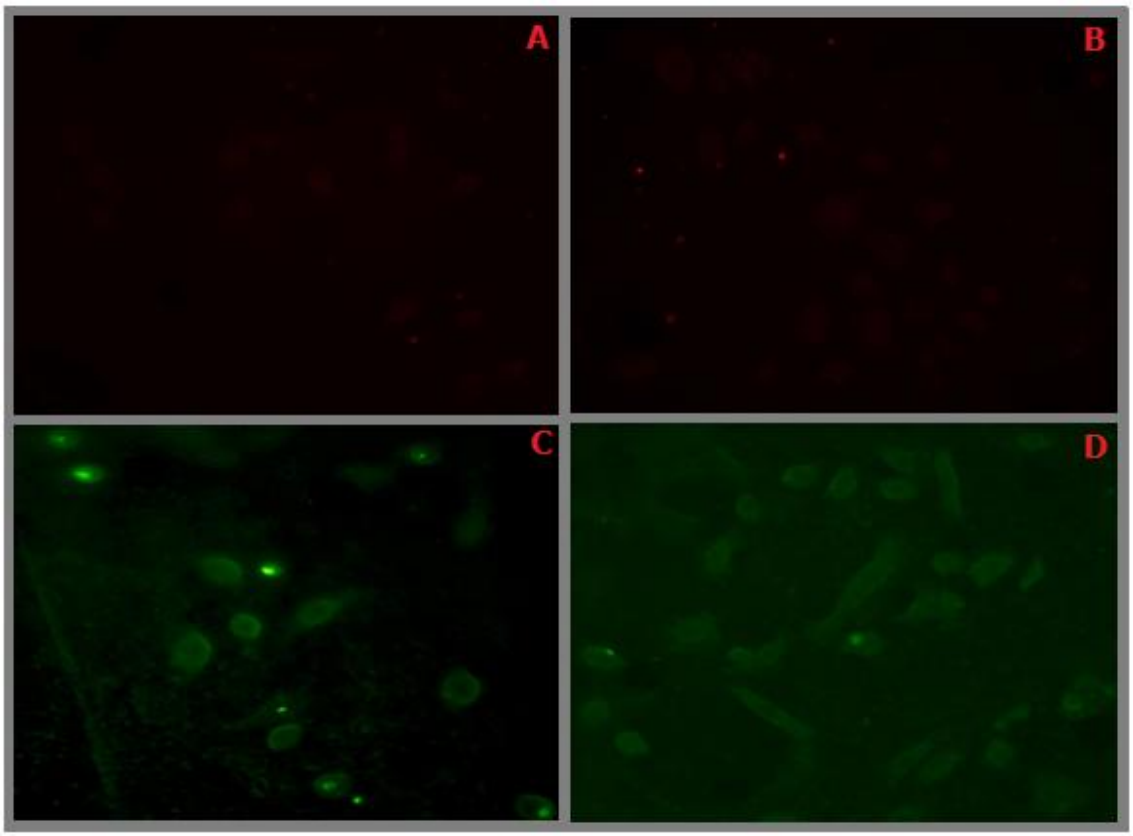

Figure 26. Cells over fibrin matrix: A) HK ß1 in fibrin TR; B) XPC ß1 in fibrin TR; C) HK α6 in fibrin DAPI; D) XPC α6 in fibrin FITC

ADHESION OVER MATRIGEL

It can be seen in figure 27.A that the healthy keratinocytes express ß1 subunit; additionally XPC cells also express this subunit as it is illustrated in figure 27.B. Respect to α6 subunit, its expression is restricted to XPC keratinocytes (figure 27.D). In contrast, healthy keratinocytes (figure 27.C) do not show any levels of expression of the α6 subunit.

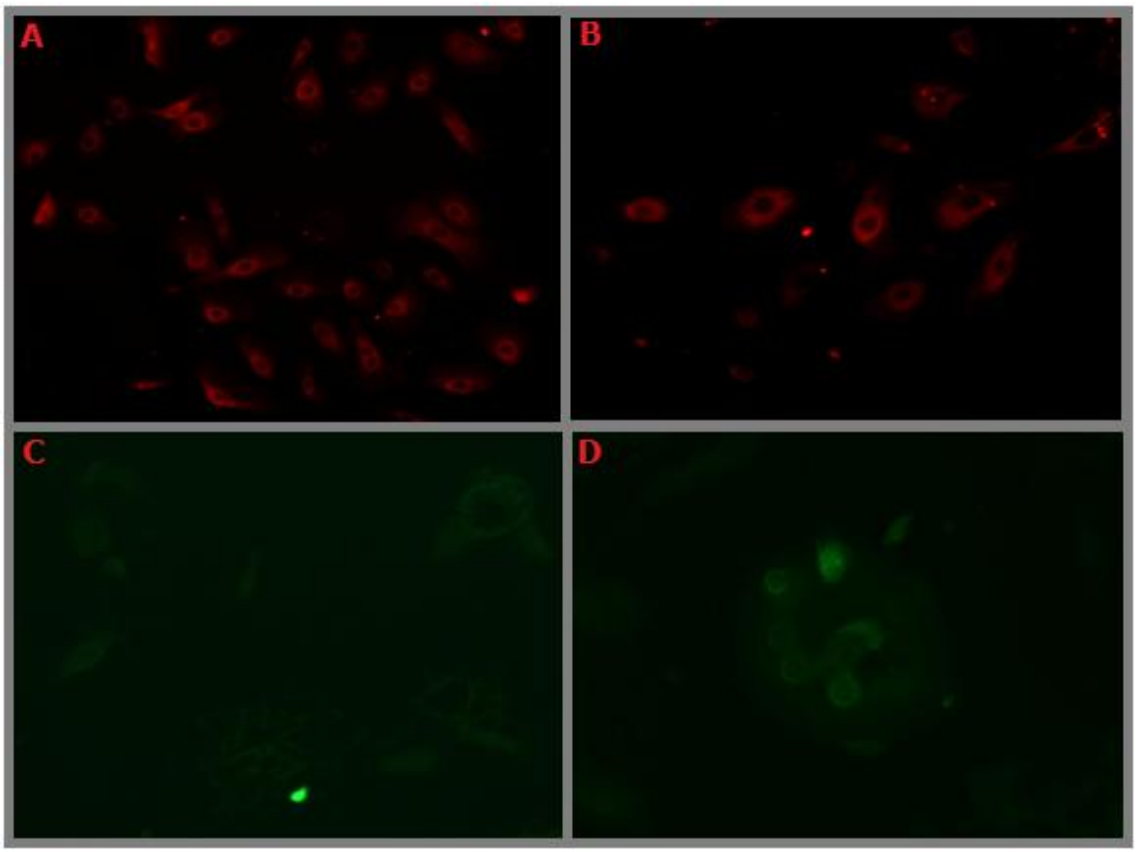

Figure 27. Cells over matrigel matrix: A) HK ß1 in fibrin TR; B) XPC ß1 in fibrin TR; C) HK α6 in fibrin DAPI; D) XPC α6 in fibrin FITC

The obtained results are unexpected because when our results are compared to Bernerd F. et al, who saw that there was an increased deposition of α6 and ß1 subunits in the basement membrane zone of XPC epidermis compared to normal epidermis at least when the experiments were done over a collagen I matrix (reconstructed in vitro) [2]. These are preliminary studies, which must be repeated to confirm the results obtained.

7.CONCLUSIONS

In conclusion, the developed studies demonstrate the high photosensitivity of XPC cells to the UVB radiation. In the sense that after a controlled dose of UVB radiation, healthy fibroblasts are able to recover completely from their DNA damage; whereas XP-C fibroblast are not able to overcome the damage, maintaining high levels of p53 protein and CPDs expression.

On the other hand, according to the performed adhesion studies, the best option is matrigel since it is the unique in which the maximum attachment of XPC keratinocytes via the integrin α6ß1 and highest cell number are found. The reason behind the highest cellular viability of matrigel is an unknown. This could have been solved by performing other experiments, which in fact will be done in the future to confirm that matrigel is the best solution as a scaffold for the possible treatment.

Finally, it should be mentioned that the obtained results “open the door” to new investigation assays; that are specified in the next section: future perspectives.

8.FUTURE PERSPECTIVES

As previously stated, other studies must be performed to obtain a better understanding of how Xeroderma Pigmentosum group C behaves at cellular level.

In the photosensitivity assay an acute damage of UVB radiation has been induced to the DNA of XPC fibroblasts and the effects have been studied by using the immunofluorescence assay. An alternative technique is Western Blot analysis to detect the specific proteins such as p53 or Ki-67. In addition, a technique called Comet assay could be used in order to detect the presence of damage in DNA.

On the other hand, respect to the adhesion study; a wide variety of matrices is available in the market including both of natural origin or artificially synthesized. Nevertheless, it is important to get a whole understanding of how is cell attachment to matrices, before choosing any other matrix as possible scaffold.

9.LEGAL REGULATORY FRAMEWORK

The present work is framed into a biomedical scientific research activity, which is recognized in Spain as a fundamental right of maximum protection in the article 20.1.b of the Spanish Constitution of 1978.

On the other hand, the specific legal regulation of biomedical investigation is found in the Law 14/2007 of July 3th; nevertheless this law has been partially modified by the Law 14/2011 of Science, technology and investigation.

At a international level, the law 14/2007 is part of the provisions of the Convention of the European Council for the protection of human rights and dignity of the human being with regard to the application of biology and medicine; It was signed in Oviedo on 14 April 1997 and entered into force in Spain 1/1/2000. In this law it is proclaimed that the health, dignity and welfare of the human being who participate in the biomedical investigation, will prevail over the interest of the society or the science.

10.SOCIOECONOMICAL IMPACT

The exact prices of all the material employed are not available at user level since they are included in the global laboratory budget. Another part of the laboratory‘s budget is reserved for the specialized equipments, which require a periodic maintenance and supervision; other costs including electronic supply are intrinsic to the use of these equipment. Some of the most common devices used in the experiment are named above:

- Thermostatized water bath "BM 302" –NÜVE
- Refrigerated centrifugator Digtor 21-R ORTOALRESA
- CO_2 incubators cell model 3517-SHEL LAB
- Conventional fluorescence microscope -BX53 OLIMPUS
- Biosafety cabinet BIO-IIA-TELSTAR

Additionally, some of the economical funds should be designated to educate all the laboratory worker in biological security as well as at least one of the members should be specialized in biological risk. Of course, all laboratories should have the appropriate security protocol against possible biological accidents in addition to proper risk label in each laboratory‘s department.

11.GLOSSARY OF TERMS

- **DNA:** deoxyribonucleic acid
- **BM:** basement membrane
- **CPDs:** cyclobutane pyrimidine dimmers
- **6-4 PPs:** 6-4 pyrimidine pyrimidone photoproducts
- **ROS**: reactive oxygen species
- **UV:** ultraviolet light
  - **UVA:** ultraviolet light type A
  - **UVB:** ultraviolet light type B
  - **UVC:** ultraviolet light type C
- **NER**: nucleotide excision repair
- **GGR**: global genomic repair
- **TCR:** transcription coupled repair
- **DMEM:**Dubelcco‘s Modified Eagle Medium
- **FBS:** fetal bovine serum
- **KCA:** keratinocytes medium

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